DEVELOPMENT OF DNA-BASED ASSAYS FOR MOLECULAR SURVEILLANCE AND POINT-OF-CARE DIAGNOSIS OF NON-FALCIPARUM MALARIA

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DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA

This dissertation is submitted to the Board of Graduate Studies, University of Ghana, in partial fulfilment of the requirements for the award of Doctor of Philosophy degree in Molecular Cell Biology of Infectious Diseases

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SEPTEMBER, 2022

DECLARATION

The experiments presented in this thesis were done by me, Felix Ansah, at the Department of Biochemistry, Cell and Molecular Biology under the supervision of Prof Gordon A. Awandare (West African Centre for Cell Biology of Infectious Pathogens and Department of Biochemistry, Cell and Molecular Biology, University of Ghana), Dr Yaw Aniweh (West African Centre for Cell Biology of Infectious Pathogens, University of Ghana) and Dr Prosper Kanyong (Siemens Healthineers, Siemens Healthcare Diagnostics Products Ltd, Llanberis, UK and West African Centre for Cell Biology of Infectious Pathogens, University of Ghana). I have duly acknowledged all references cited in this study.

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ACKNOWLEDGEMENT

My most profound appreciation to all the individuals and their parents/guardians who consented to participate in the study.

I am deeply indebted to my supervisor and mentor, Prof Gordon A. Awandare, for all the guidance and opportunities he offered me throughout my study. I am also grateful to Dr Yaw Aniweh and Dr Prosper Kanyong for their supervision and invaluable inputs in the study. It is your love and support that made this study successful.

I appreciate the support of all members of the Infectious Disease Research Laboratory of the Department of Biochemistry, Cell and Molecular Biology, especially Dr Francis Krampa and Mr Jacob Donkor.

I am also grateful to the staff of Ewim Polyclinic, Richard Novati Catholic Hospital and Kintampo Health Research Centre for their help with the sample collection. Lastly, I would like to thank the TIBA-Ghana team and the TIBA-Ghana local expert advisory group for their invaluable contributions.

The study was funded by the National Institute for Health Research (NIHR) Global Health Research Unit grant for Tackling Infections to Benefit Africa (16/136/33: Woolhouse, M) to the University of Edinburgh, using UK aid from the UK Government, the World Bank African Centres of Excellence for Development Impact grant (ACE IMPACT - WACCBIP-NCDs: Awandare, G) and the Wellcome/African Academy of Sciences DELTAS Africa grant (DEL-15-007: Awandare, G).

DEDICATION

To my parents, Mr Bismarck Ansah and Mrs Georgina Ansah. I love you.

I also dedicate this work to all the wonderful people, teachers, and lecturers who supported and contributed in diverse ways towards my growth from the basic level to the apex of my academic journey.

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LIST OF ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
AOR	Adjusted odds ratio
ATCC	American type culture collection
BLAST	Basic local alignment search tool
Bst-LF	*Bacillus stearothermophilus* Large Fragment
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
C_t-value	Threshold cycle value
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
CV	Cyclic voltammetry
EIS	Electrochemical impedance spectroscopy
IBM-SPSS	International business machines-statistical package for the Social Sciences
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
MCH	Mercaptohexanol
NAATs	Nucleic acid amplification tests
NCBI	National Centre for Biotechnology Information
PBS	Phosphate-buffered saline
PfGDH	*P. falciparum* glutamate dehydrogenase
PfHRP2	*P. falciparum* histidine-rich protein II
PfLDH	*P. falciparum* lactate dehydrogenase

pLDH	Pan *Plasmodium* lactate dehydrogenase
POC	Point-of-care
QCM	Quartz crystal microbalance
qPCR	Quantitative polymerase chain reaction
RBCs	Red blood cells
RDTs	Rapid diagnostic tests
rRNA	Ribosomal ribonucleic acid
RT-LAMP	Real-time loop-mediated isothermal amplification
SERS	Surface-enhanced Raman scattering/spectroscopy
SPR	Surface plasmon resonance
SYBR	Synergy brands
Taq	*Thermus aquaticus*
TE	Tris-ethylenediamine tetraacetic acid
WBCs	White blood cells
WHO	World Health Organisation

LIST OF SYMBOLS AND UNITS OF MEASUREMENT

~	Approximately
χ^2	Chi-Square value
^oC	Degree Celsius
=	Equal to
>	Greater than
<	Less than
r	Pearson's correlation coefficient
R^2	Coefficient of determination
%	Per cent
±	Plus or minus
P	Probability value
RR	Relative response
R_{ct}	Resistance to charge transfer
rpm	Rotation per minute
S	Seconds
Σ	Standard deviation
Cm	Centimetre
aM	Attomolar
fM	Femtomolar
pM	Picomolar
G	Gram
Mg	Microgram
Ng	Nanogram

Pg	Pictogram
µL	Microlitre
mL	Millilitre
mM	Millimolar
kDa	Kilo Dalton
mV	Millivolt
MΩ	Mega Ohms
µA	Microampere
kHz	Kilohertz

ABSTRACT

Background

In recent years, the clinical significance of *Plasmodium malariae* and *Plasmodium ovale* is increasingly gaining public health attention as the global transmission of falciparum malaria is decreasing. However, the most readily available and cost-effective malaria diagnostic tools, which include microscopy and antigen-based rapid diagnostic tests, lack adequate sensitivity and specificity for accurate surveillance and point-of-care diagnosis of these non-falciparum species. This challenge poses a major setback to global efforts aimed towards malaria control and elimination. In this study, DNA-based assays with improved sensitivity and specificity were developed for species-specific detection of *P. malariae* and *P. ovale*.

Methods

SYBR Green-based real-time quantitative polymerase chain reaction (qPCR) assays were developed for the detection of *P. malariae* and *P. ovale* using a new set of primers called cooperative primers. The cooperative primer-based qPCR assays were used in a cross-sectional study to determine the prevalence rates of *P. malariae* and *P. ovale* using field samples obtained from three malaria transmission settings in Ghana. Following this, the associations between *P. malariae* and *P. ovale* infections and haematological indices were assessed. For point-of-care diagnosis of *P. malariae* and *P. ovale* associated malaria, in-house real-time loop-mediated isothermal amplification (RT-LAMP) assays were developed and the diagnostic performance of the RT-LAMP assays were compared to the cooperative primer-based qPCR assays. In addition, label-free DNA-based electrochemical biosensors were developed for ultrasensitive detection of *P. malariae* and *P. ovale*.

Results

Both the *P. malariae* and the *P. ovale* cooperative primer-based qPCR assays had a detection limit of approximately 1.0 parasite/µL, which is at least 10-fold lower than the corresponding conventional primer-based qPCR assays. Using the cooperative primer-based qPCR assays in a cross-sectional study, the prevalence rates of *P. malariae* and *P. ovale* infections among the combined study population were 13.3% and 4.8%, respectively. Notably, study participants harbouring mixed infections of *P. falciparum* with either *P. malariae* or *P. ovale* had the greatest risk of developing anaemia. The diagnostic sensitivity and specificity of the in-house RT-LAMP assays were in the range of 86.2% - 97.5% when compared to the cooperative primer-based qPCR assays. Remarkably, both the *P. malariae* and the *P. ovale* DNA-based biosensors showed a sensitivity of 100% using purified genomic DNA samples. However, the specificities of the biosensors were 100% and 66.7% for *P. malariae* and *P. ovale*, respectively.

Conclusion

In summary, the results demonstrate that the DNA-based assays described in this study have adequate sensitivity and specificity for the detection of *P. malariae* and *P. ovale* in clinical isolates. This study highlights the importance of including detection tools with lower detection limits in the routine surveillance and point-of-care diagnosis of non-falciparum species. The availability of reliable and cost-effective species-specific detection tools for *P. malariae* and *P. ovale* will be necessary for comprehensively assessing the effectiveness of malaria interventions and control measures aimed towards global malaria elimination.

CHAPTER ONE

1.1 GENERAL INTRODUCTION

Plasmodium malariae and *Plasmodium ovale* are less prevalent human *Plasmodium* species that are generally associated with milder forms of malaria (Mueller *et al*., 2007). These less common "minor" non-falciparum species are usually detected as low-density infections and co-infections with the dominant *Plasmodium falciparum* and *Plasmodium vivax* (Collins & Jeffery, 2005, 2007; Hawadak *et al*., 2021). Over the past decades, malaria interventions and control measures have mainly focused on *P. falciparum* and *P. vivax* with the other non-falciparum species, being neglected due to their marginal contribution to global malaria burden (Hawadak *et al*., 2021; Weiss *et al*., 2019; World Health Organisation, 2015). However, recent studies have highlighted the clinical significance of these non-falciparum species (Douglas *et al*., 2013; Langford *et al*., 2015). *P. malariae* and *P. ovale* have been implicated in major disease conditions such as severe anaemia, kidney-related complications, respiratory distress, hypotension, severe thrombocytopenia, jaundice, hepatomegaly, and hepatic dysfunction with likely fatal outcomes (Rojo-Marcos *et al*., 2008; Douglas *et al*., 2013; Silva *et al*., 2017; D'Abramo *et al*., 2018; Gentile *et al*., 2019; Kotepui *et al*., 2020a). In addition, recent reports indicate an increasing prevalence of *P. malariae* and *P. ovale* in areas where *P. falciparum* transmission is decreasing (Betson *et al*., 2018; Gnémé *et al*., 2013; Yman *et al*., 2019). Therefore, the availability of reliable tools for timely and accurate detection of *P. malariae* and *P. ovale* will help inform appropriate antimalarial treatment and the implementation of effective control measures.

Currently, the most readily available and cost-effective detection tools for malaria diagnosis include microscopy and antigen-based rapid diagnostic tests (RDTs) (Krampa *et al*., 2017; World Health Organisation, 2020). However, the application of these tools for the detection of

P. malariae and *P. ovale* has been limited by the morphological similarities among *Plasmodium* species and the characteristic low-density infections of these non-falciparum species (Gimenez *et al*., 2021; Mueller *et al*., 2007). As such, nucleic acid-based amplification tests (NAATs) are generally considered as the most reliable tools for the detection of *P. malariae* and *P. ovale* (Mueller *et al*., 2007; Tangpukdee *et al*., 2009). Among the available NAATs, polymerase chain reaction (PCR) is the most commonly used tool for detecting *Plasmodium* species (Tangpukdee *et al*., 2009). Despite the high analytical performance of PCR-based assays, accurate detection of low-density infections in field isolates remains a challenge (Amaral *et al*., 2019; Hofmann *et al*., 2015; Satterfield, 2014). This limitation is partly due to the formation of non-specific products such as primer-dimers which are commonly associated with the amplification of lower concentrations of target nucleic acid (Chou *et al*., 1992; Satterfield, 2014). In addition, the use of PCR-based assays at the point-of-care (POC) has been hampered by the operational requirements and the high cost, which are beyond the capacity of most malaria-endemic countries (Lucchi *et al*., 2010).

An alternative NAAT tool that has been described for the diagnosis of *P. malariae* and *P. ovale* at the POC is loop-mediated isothermal amplification (LAMP) (Han *et al*., 2007). Compared to PCR, LAMP is a relatively quick and simple technique with minimal operational requirements (Becherer *et al*., 2020; Thompson & Lei, 2020). This field-adaptable technique amplifies target nucleic acid under isothermal conditions (constant temperature) with high specificity and sensitivity using *Bacillus stearothermophilus* (*Bst*) polymerase, a DNA polymerase with strand displacement activity (Becherer *et al*., 2020). Several LAMP assays have been reported for the detection of *Plasmodium* species with limits of detection of less than 10 parasites/µL of blood (Hopkins *et al*., 2013; Lau *et al*., 2016; Mohon *et al*., 2014; Polley *et al*., 2010). Despite these advantages, the routine application of LAMP at the POC has also been hampered due to the high cost of LAMP assay reagents, particularly the *Bst* polymerase

(Becherer *et al*., 2020). As such, the development of LAMP assays using locally produced *Bst* polymerase would be a major step towards the development of cost-effective and reliable diagnostics for non-falciparum malaria at the POC.

Another diagnostic field that has gained public health attention due to the increasing demand for reliable POC diagnostic devices is the biosensing platform (Rodovalho *et al*., 2015). The use of biosensors, particularly the electrochemical-based ones, for POC applications for monitoring blood glucose levels have a history that spans several decades (Wang, 2008). Currently, several biosensors have been reported for malaria diagnosis (Krampa *et al*., 2020). However, most of these biosensors employ *P. falciparum* histidine-rich protein II (PfHRP2) and genus *Plasmodium* lactate dehydrogenase (pLDH) antigenic biomarkers, which lack adequate specificity and sensitivity for species-specific detection of *P. malariae* and *P. ovale* (Agaba *et al*., 2019; Simpalipan *et al*., 2018; Verma *et al*., 2018). Alternatively, nucleic acid-based biosensors provide relatively highly sensitive and specific diagnosis (Cordray & Richards-Kortum, 2012). In addition, biomarkers and biorecognition receptors for nucleic acid-based biosensors are relatively easy to identify, cost-effective, and have high stability (Morales & Halpern, 2018; Teles & Fonseca, 2008). Despite these advantages, there is little advancement in the development of nucleic acid-based biosensors for the diagnosis of malaria.

In this study, real-time quantitative PCR (qPCR) assays were developed for the detection of *P. malariae* and *P. ovale* using cooperative primers that significantly limit the formation and propagation of primer-dimers (Ansah *et al*., 2021). Following this, the new cooperative primer-based qPCR assays were used in a cross-sectional study to determine the prevalence of *P. malariae* and *P. ovale* using samples obtained from three malaria transmission settings in Ghana and the associations between these non-falciparum species and haematological parameters were assessed. Towards the development of cost-effective and reliable POC diagnostic tools, in-house real-time LAMP (RT-LAMP) assays were developed for the

detection of *P. malariae* and *P. ovale* using locally produced *Bst* polymerase. In addition, the study describes the first DNA-based electrochemical biosensors that allowed for species-specific ultrasensitive detection of *P. malariae* and *P. ovale* in clinical isolates without the need for pre-amplification of target DNA (Ansah *et al*., 2022).

1.2 SPECIFIC AIMS

1.2.1 Specific Aim 1

To develop cooperative primer-based qPCR assays for the detection of *P. malariae* and *P. ovale*.

1.2.2 Specific Aim 2

a) To determine the prevalence of *P. malariae* and *P. ovale* in different malaria transmission settings in Ghana.

b) To determine the associations between *P. malariae* and *P. ovale* infections and haematological parameters.

1.2.3 Specific Aim 3

To develop in-house RT-LAMP assays for cost-effective nucleic acid-based diagnosis of *P. falciparum, P. malariae* and *P. ovale* at the point-of-care (POC).

1.2.4 Specific Aim 4

To develop label-free DNA-based electrochemical biosensors for the detection of *P. falciparum, P. malariae* and *P. ovale* in clinical isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The burden of human malaria

Human malaria remains a major global health concern despite the introduction of several interventions and control measures over the past two decades (World Health Organisation, 2020). In 2019, a higher number of malaria cases of 229 million were reported compared to the total malaria cases of 225 million recorded in 2009 (World Health Organisation, 2020). Globally, it is estimated that nearly half a million malaria-associated deaths occur annually (World Health Organisation, 2020). Notably, sub-Saharan Africa disproportionately shares a greater burden of the disease accounting for ~94% of all malaria cases and ~95% malaria deaths in 2019, with children under five years and pregnant women being the most vulnerable groups (World Health Organisation, 2020). In Ghana, malaria continues to be one of the leading diseases accounting for ~40% of all outpatient cases (Ameme *et al*., 2014). In 2019, a total estimate of ~5 million malaria cases and ~11,000 malaria-associated deaths were reported in Ghana, which represents ~2% global malaria cases and ~3% global malaria deaths, respectively (World Health Organisation, 2020). These relative high proportions of malaria cases and deaths in Ghana and Africa at large necessitate the availability of rapid, cost-effective and reliable malaria diagnostic tools.

2.2 Human malaria *Plasmodium* species

Malaria is caused by protozoan parasites of the genus *Plasmodium*. A total of five distinct *Plasmodium* species have been implicated in human malaria, namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* (White, 2008). Among these species, *P. falciparum* and *P. vivax* account for the vast majority

of malaria morbidity and mortality (World Health Organisation, 2020). *P. falciparum* is the most dominant *Plasmodium* species and causes a wide spectrum of clinical manifestations ranging from mild to life-threatening severe malaria (Bartoloni & Zammarchi, 2012). In 2019, *P. falciparum* accounted for more than 99% of malaria cases in sub-Saharan Africa, 71% in the Eastern Mediterranean, 65% in the Western Pacific and 50% in South-East Asia (World Health Organisation, 2020). *P. vivax* is most prevalent in Asia and Latin America, accounting for more than 75% of malaria cases outside Africa (World Health Organisation, 2020). Until recently, *P. vivax* was commonly thought to infect host red blood cells using the Duffy antigen. As such, individuals lacking Duffy antigen, a phenomenon common in West Africa, are largely refractory to *P. vivax* infection (Livingstone, 1984). However, recent findings indicate that *P. vivax* infection is common in Duffy-negative phenotype (Howes *et al*., 2011), with epidemiological evidence of *P. vivax* transmission in Duffy-negative individuals reported in many parts of Africa, including Nigeria (Oboh *et al*., 2020), Congo (Brazeau *et al*., 2018), Uganda (Dhorda *et al*., 2011), Cameroon (Dongho *et al*., 2021), Mali (Niangaly *et al*., 2017) and Angola (Mendes *et al*., 2011). In Ghana, no case of *P. vivax* infection has been reported (Amoah *et al*., 2019; Brown *et al*., 2021). *P. knowlesi* was initially known to cause only simian malaria; however, it is now considered the fifth human *Plasmodium* species (White, 2008). Since the first report of *P. knowlesi* infection in Malaysia in 2004, almost all countries in Southeast Asia have reported cases of *P. knowlesi* infection (Singh & Daneshvar, 2013). However, *P. knowlesi* infection has not yet been reported in Africa (Amir *et al*., 2018). The other non-falciparum *Plasmodium* species, *P. malariae* and *P. ovale*, are less common but have been reported in all malaria-endemic regions. These less common non-falciparum species cause milder malaria than the dominant *P. falciparum* and *P. vivax* (Mueller *et al*., 2007).

2.3 *P. malariae* and *P. ovale* infections

P. malariae was the first human malaria parasite to be described in the late 19th century (Collins & Jeffery, 2007). *P. malariae* parasites have a predilection for matured red blood cells (RBCs) (Garnham, 1966). Following RBC invasion, the parasite accumulates hemozoin and forms characteristic pink stains called Ziemann's dots (Taylor & Agbenyega, 2012). Morphologically, the *P. malariae* appears as a band across the infected RBC at the trophozoite stage and resembles mega-gametocytes after maturation (Bogitsh *et al*., 2018). In addition, *P. malariae* appears to have no modification in RBC size or diameter after invasion, which may be due to the parasite's preference for matured erythrocytes (Bogitsh *et al*., 2018). The schizogony of *P. malariae* infection synchronously occurs every 72 hours, which is a distinctive phenomenon associated with *P. malariae* infection that is commonly referred to as quartan malaria (Collins & Jeffery, 2007).

P. ovale was first reported in 1922 by Stephens (Stephens, 1922). Contrary to *P. malariae*, *P. ovale* parasites have an affinity for immature RBCs (Brazeau *et al*., 2018). Morphologically, the trophozoite stage of the parasite is characterised by an irregular and amoeboid-like shape (Bogitsh *et al*., 2018). In addition, *P. ovale* infection results in enlargement of infected RBCs resulting in a characteristic ellipsoid shape (Collins & Jeffery, 2005). The duration for successive *P. ovale* schizogony is approximately 48 hours which causes a condition called tertian malaria (Bogitsh *et al*., 2018). Currently, *P. ovale* has been sub-classified into two sympatric non-recombinant subspecies, namely *P. ovale wallikeri* and *P. ovale curtisi* (Sutherland *et al*., 2010).

2.4 The global distribution of *P. malariae* and *P. ovale*

P. malariae and *P. ovale* are less prevalent non-falciparum *Plasmodium* species (Hawadak *et al*., 2021; Mueller *et al*., 2007). Although found in all malaria-endemic regions, these "minor" non-falciparum species are most prevalent in sub-Saharan Africa (Hawadak *et al*., 2021). PCR-based cross-sectional studies in Ghana, Nigeria, Uganda, Angola, Kenya, Malawi and Equatorial Guinea have reported prevalence ranging from 1% to 30% for both *P. malariae* and *P. ovale* (Amoah *et al*., 2019; Bruce *et al*., 2008; Dinko *et al*., 2013; Fançony *et al*., 2012; Oboh *et al*., 2018; Oguike *et al*., 2011; Owusu *et al*., 2017). Other studies in Asia and South America have also reported prevalence ranging from 1% to 37% for these non-falciparum species (Mehlotra *et al*., 2000; Mueller *et al*., 2007). In recent years, several epidemiological studies have observed an increase in the prevalence of *P. malariae* and *P. ovale* in parts of Africa, South America and Asia (Camargo *et al*., 2018; Guerra *et al*., 2019; Sitali *et al*., 2019; Taylor *et al*., 2011; Woldearegai *et al*., 2019). Interestingly, a more recent study in Nigeria observed an unexpectedly high prevalence of 66.4% and 30.5% for *P. malariae* and *P. ovale*, respectively (Abdulraheem *et al*., 2022). It is worth noting that the prevalence of both *P. malariae* and *P. ovale* varies among different study populations and across various study sites (Hawadak *et al*., 2021; Mueller *et al*., 2007).

For global trend analysis, a recent study comprising of selected data published from 2000 to 2020 observed that the overall prevalence of *P. malariae* and *P. ovale* were 2.01% and 0.77%, respectively (**Figure 1**) (Hawadak *et al*., 2021). The study also observed a reduction in the prevalence of these non-falciparum species over the last two decades, although the reduction was not statistically significant (Hawadak *et al*., 2021). These findings parallel another longitudinal study from 1990 to 2010 in Senegal, where *P. malariae* and *P. ovale* infections contributed to less than 6% of all malaria cases, and decreased trends were observed for both *P. malariae* and *P. ovale* infections (Roucher *et al*., 2014). Contrary to these reports, a study

conducted over a period of 22 years (1994 - 2016) in Tanzania reported a two-fold and a six-fold increase in the prevalence of *P. malariae* and *P. ovale*, respectively (Yman *et al*., 2019).

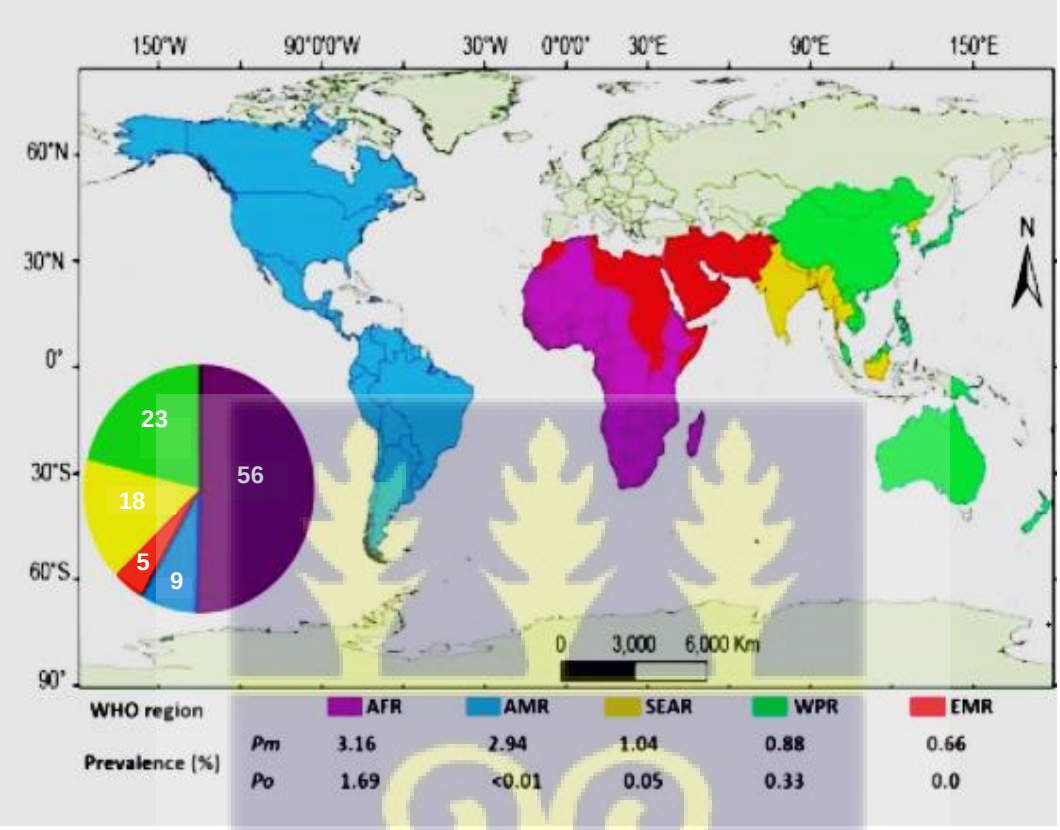

Figure 1: The global distribution of *P. malariae* and *P. ovale.*

The average prevalence of *P. malariae* (*Pm*) and *P. ovale* (*Po*) based on selected studies from 2000 to 2020 for the World Health Organisation African Region (AFR), Region of the Americas (AMR), South-East Asia Region (SEAR), Western Pacific Region (WPR), and Eastern Mediterranean Region (EMR). The numbers in the pie chart represent the number of included studies for each region (Source: Hawadak *et al*., 2021).

2.5 The clinical manifestation and significance of *P. malariae* and *P. ovale*

2.5.1 Clinical symptoms

Malaria associated with *P. malariae* and *P. ovale* is generally considered "benign" (Mueller *et al*., 2007; Strydom *et al*., 2014). These non-falciparum species are associated with milder malaria compared to *P. falciparum* and *P. vivax*. In some cases, *P. malariae* and *P. ovale* malaria are self-limiting without treatment (Bogitsh *et al*., 2018). However, some recent studies have implicated these less common non-falciparum species in major disease burden such as severe anaemia, kidney-related complications, respiratory distress, hypotension, severe thrombocytopenia, and petechiae (Rojo-Marcos *et al.*, 2008; Douglas *et al.*, 2013; Langford *et al.*, 2015; D'Abramo *et al.*, 2018; Gentile *et al.*, 2019; Kotepui *et al.*, 2020a). In addition, cases of liver-related complications that result in jaundice, hepatomegaly and hepatic dysfunction with fatal outcomes have been reported (Silva *et al*., 2017).

2.5.2 Relapses and recrudescence

Both *P. malariae* and *P. ovale* cause long-lasting asymptomatic chronic infections (Mueller *et al*., 2007). *P. ovale* infection results in the formation of dormant hypnozoites in the liver cells (Collins & Jeffery, 2005). These hypnozoites, if not treated, could periodically relapse and cause life-long infection (Bogitsh *et al*., 2018). Unlike *P. ovale*, *P. malariae* infection does not cause relapse infection since *P. malariae* parasites do not produce exoerythrocytic dormant hypnozoites (Collins & Jeffery, 2007). However, several cases of *P. malariae* recurring infections have been reported (Chadee *et al*., 2000; Grande *et al*., 2019; Lubis *et al*., 2020; Vinetz *et al*., 1998). This observation has been associated with a sudden increase in persistent sub-patent *P. malariae* parasites in the blood, which is a phenomenon known as recrudescence

(Collins & Jeffery, 2007). Notably, a clinical case of *P. malariae* recrudescence after 53 years of initial infection has been reported (Guazzi & Grazi, 1963).

2.5.3 Antimalarial treatment

Several studies have evaluated the efficacy of various antimalarial drugs against *P. malariae* and *P. ovale*. Generally, *P. malariae* and *P. ovale* infections are sensitive to chloroquine (Mockenhaupt *et al*., 2000; World Health Organisation, 2015). Some other antimalarial drugs recommended for the treatment of *P. malariae* and *P. ovale* infections include atovaquone-proguanil, mefloquine and quinine sulfate plus doxycycline or tetracycline (Avina-Zubieta & Esdaile, 2018; Hill *et al*., 2021; World Health Organisation, 2015). For *P. ovale* infection, a radical treatment approach that requires primaquine or tafenoquine in addition to the regular treatment is recommended to treat the dormant liver-stage hypnozoites (World Health Organisation, 2015). It is worth noting that almost all clinical malaria are treated with the first-line artemisinin-based combination therapies (ACTs), which are generally considered to be effective against both falciparum and non-falciparum *Plasmodium* species (World Health Organisation, 2015). However, several cases of *P. malariae* and *P. ovale* recurring infections have been reported after treatment with ACTs (Betson *et al*., 2014, 2018; Dinko *et al*., 2013; Rutledge *et al*., 2017). This observation calls for the introduction of reliable species-specific tools for malaria diagnosis at the POC.

2.6 Detection methods for *P. malariae* and *P. ovale*

Accurate detection of non-falciparum species is critical for disease management and malaria control. Currently, the available malaria diagnostic tools include microscopy, rapid diagnostic tests (RDTs), and nucleic acid amplification tests (NAATs) (Krampa *et al*., 2017; World Health Organisation, 2020). Among these, microscopy and RDTs are the most readily available and cost-effective tools. However, the limited sensitivity and poor specificity of these tools have been a major setback for accurate detection of *P. malariae* and *P. ovale* in clinical samples (Gimenez *et al*., 2021; Snounou *et al*., 1993a). Alternatively, NAATs such as PCR and LAMP provide relatively high sensitive and specific diagnosis (Han *et al*., 2007; Hofmann *et al*., 2015; Polley *et al*., 2010). Both PCR and LAMP assays have been reported for the detection of *P. malariae* and *P. ovale* (Han *et al*., 2007; Rougemont *et al*., 2004; Snounou *et al*., 1993b). However, the routine application of NAATs has also been hampered by several factors, including the high cost of reagents, expensive equipment, and the need for well-trained personnel (Becherer *et al*., 2020). The current diagnostic tools for *P. malariae* and *P. ovale*, the associated challenges and the advances in the development of new diagnostic tools have been further discussed in subsequent sections.

2.6.1 Microscopy

Microscopy is based on the examination of parasite morphology on Giemsa-stained thick and thin blood smears (Fleischer, 2004). This detection technique is readily available, cost-effective and requires minimal infrastructure (Bailey *et al*., 2013; CDC, 2018). Microscopy is usually considered the gold standard for malaria parasite identification and provides species-specific qualitative and quantitative data (Bailey *et al*., 2013; CDC, 2018; Wilson, 2013). The limit of detection of microscopy is within the range of 50 – 100 parasites/µL of blood (Britton *et al*.,

2016; malERA Consultative Group on Diagnoses and Diagnostics, 2011). Despite these advantages, several factors are known to affect the accuracy of microscopy for the detection of *P. malariae* and *P. ovale* (O'Meara *et al*., 2005, 2006). Among these factors are the technical expertise required (Durrheim *et al*., 1997; Maguire *et al*., 2006), microscope and slide quality (World Health Organisation, 2016), and the characteristic low-density infections of non-falciparum species. In addition to these factors, *P. malariae* and *P. ovale* are morphologically similar to *P. falciparum* and *P. vivax*, respectively (Lee *et al*., 2009; Kotepui *et al*., 2020b). As such, *P. malariae* and *P. ovale* are commonly misdiagnosed as the other *Plasmodium* species, especially in cases of mixed infections with *P. falciparum* and *P. vivax* (Lee *et al*., 2009; Barber *et al*., 2013a; Kotepui *et al*., 2020b). A recent meta-analysis study of PCR-confirmed *P. ovale* cases observed that a pooled prevalence of 11% of *P. ovale* cases was misdiagnosed as *P. vivax* (Kotepui *et al*., 2020b).

The prevalence of *P. malariae* and *P. ovale* reported in studies as determined by microscopy indicate a varying range of 0% - 37% depending on the geographical location and demographic characteristics of the study group (Cavasini *et al*., 2000; Ibekwe *et al*., 2009; Mehlotra *et al*., 2000; Noland *et al*., 2014; Rubio *et al*., 1999; Snounou *et al*., 1993a; Walker-Abbey *et al*., 2005; Zhou *et al*., 1998). In epidemiological studies comparing microscopy to other detection methods such as PCR, it was observed that a significant proportion of *P. malariae* and *P. ovale* infections are missed by microscopy (Mueller *et al*., 2007). This observed under-diagnosis of non-falciparum by microscopy is quite frequent in non-endemic settings where laboratories are not familiar with the morphology of these less prevalent *Plasmodium* species (Edson *et al*., 2010). In one study on the proficiency test on the identification of *Plasmodium* species, it was observed that most of the participating laboratories were proficient in identifying *P. falciparum*, but not *P. malariae* and *P. ovale* (Edson *et al*., 2010). Taken together, although microscopy has several advantages, the poor sensitivity and specificity in the identification and

differentiation of *Plasmodium* species could, to a large extent, negatively impact malaria management and control measures, especially in the recommendation of appropriate antimalarial drugs (World Health Organisation, 2015).

2.6.2 Malaria rapid diagnostic tests (RDTs)

Malaria RDTs are immunochromatographic tests that are based on antigen-antibody interaction (Wongsrichanalai *et al*., 2007). Similar to microscopy, malaria RDTs are widely employed in most clinical and laboratory settings (Tangpukdee *et al*., 2009; Wongsrichanalai *et al*., 2007; World Health Organisation, 2020). RDTs are portable, disposable devices that require minimal operational training (Tangpukdee *et al*., 2009). In addition, RDTs are economical and require no laboratory infrastructure, instruments, or electricity (Wongsrichanalai *et al*., 2007). Currently, the available malaria RDTs employed biomarkers that are specific for *P. falciparum* and *P. vivax*, or genus *Plasmodium* with *P. falciparum* histidine-rich protein II (PfHRP2) and genus *Plasmodium* lactate dehydrogenase (pLDH) being the most commonly targeted biomarkers (Krampa *et al*., 2017).

PfHRP2 is usually used alone or in combination with pLDH (Tangpukdee *et al*., 2009; Wongsrichanalai *et al*., 2007; World Health Organisation, 2020). Several PfHRP2-based RDT kits with varying sensitivity and specificity are available from different manufacturers (Omondi *et al*., 2017; Rosenthal, 2012). The observed variations in the analytical performance of RDTs have been partly attributed to differences in manufacturing processes, storage conditions, and study design (Chiodini, 2014; Chiodini *et al*., 2007; Mouatcho & Goldring, 2013). A number of studies using PfHRP2/pLDH-based RDTs from different manufacturers have reported sensitivity and specificity in the range of 29% - 100% and 56.0% - 89.0%, respectively (Barber *et al*., 2013b; Foster *et al*., 2014; Maltha *et al*., 2014; Alareqi *et al*., 2016; Ogunfowokan *et al*.,

2020). Of note, RDTs generally have low efficiency for the diagnosis of low-density infections that are below 200 parasites/µL (Hopkins *et al*., 2007; McMorrow *et al*., 2011).

A number of factors that limit RDTs for accurate species-specific diagnosis of malaria have been reported. Firstly, the sensitivity of the existing RDTs is affected by genetic variation in PfHRP2 and pLDH antigens. Different studies have reported an extensive diversity in the genomic sequence of PfHRP2 and pLDH among parasite populations within and across countries (Agaba *et al*., 2019; Baker *et al*., 2005, 2010; Simpalipan *et al*., 2018; Verma *et al*., 2018). Secondly, the persistence of PfHRP2 antigen in blood circulation weeks after parasite clearance could result in false positives (Dalrymple *et al*., 2018; Iqbal *et al*., 2004; Mayxay *et al*., 2001). In addition, the performance of antigen-based RDTs for the detection of *P. malariae* and *P. ovale* based on the pan *Plasmodium* antigen (pLDH) is relatively poor, with sensitivity ranging from 0.0% to 85.7% (Grobusch *et al*., 2002; Haanshuus *et al*., 2016; Houzé *et al*., 2011; Playford & Walker, 2002; Tanizaki *et al*., 2014; Van-Dijk *et al*., 2009; Yerlikaya *et al*., 2018). These, among other factors, limit the reliability of antigen-based RDTs for species-specific diagnosis of malaria (Ranadive *et al*., 2017). As such, nucleic acid amplification tests (NAATs) are usually recommended for the detection of *P. malariae* and *P. ovale* infections (Gimenez *et al*., 2021).

2.6.3 Nucleic acid amplification tests (NAATs)

2.6.3.1 Polymerase chain reaction (PCR)

PCR is a NAAT that has been widely used for the detection of *Plasmodium* species using various sample types such as blood, urine and saliva (Buppan *et al*., 2010; Johnston *et al*., 2006; Tedla, 2019). PCR involves the amplification of specific RNA and/or DNA sequences to a detectable level with high sensitivity and specificity (Mullis, 1990; Valones *et al*., 2009). This technique provides reliable assays with substantially improved analytical performance for the

detection of low-density non-falciparum infections compared to microscopy and RDTs (Tedla, 2019). The first PCR assays for the detection of *P. malariae* and *P. ovale* were developed in 1993 (Snounou *et al*., 1993b). These were nested PCR assays that target the 18S small subunit ribosomal RNA (18S rRNA) gene with limit of detection of 10 parasites/µL for both *P. malariae* and *P. ovale* (Snounou *et al*., 1993b). Since this development, several other nested PCR assays have been reported for the detection of these non-falciparum species, most of which target the 18S rRNA gene due to its relatively high copy number, 5-8 copies/genome (Amaral *et al*., 2019; Rubio *et al*., 2002). However, nested PCR is time-consuming, expensive, and prone to cross-contamination since it requires two PCR steps (Tedla, 2019). As such, real-time quantitative (qPCR) assays involving the use of fluorescent dyes for single-step amplification have been described for the detection of *P. malariae* and *P. ovale* (Gimenez *et al*., 2021). These single-step qPCR assays include multiplex qPCR assays (Chew *et al*., 2012; Rougemont *et al*., 2004; Shokoples *et al*., 2009; Veron *et al*., 2009) and monoplex qPCR assays (Mangold *et al*., 2005). Multiplex qPCR allows simultaneous detection of different *Plasmodium* species in a single reaction using multiple primer sets, which make the technique relatively economical for species-specific diagnosis of malaria (Gimenez *et al*., 2021; Markoulatos *et al*., 2002). However, due to the disproportional ratio of high-density *P. falciparum* to low-density non-falciparum *Plasmodium* species, there is the risk of missing the low-density non-falciparum species (Gimenez *et al*., 2021; Obare *et al*., 2013). In addition, the use of multiple primers exponentially increases the risk of non-specific amplification resulting in the formation of non-specific products such as primer-dimers which are commonly associated with the detection of low-density infections (Poritz & Ririe, 2014; Satterfield, 2014). As such, several attempts have been made to develop methods, including hot starts and modified primers, that hinder or limit the formation of non-specific products (Birch, 1996; Chou *et al*., 1992; Kainz *et al*., 2000; Lebedev *et al*., 2008; Satterfield, 2014). Among these

developments, cooperative primer-based technology is the first approach that simultaneously limits the formation and the propagation of primer-dimers (Poritz & Ririe, 2014; Satterfield, 2014). A cooperative primer-based qPCR assay has been reported for the detection of *P. falciparum* (Satterfield, 2014). The cooperative primer-based qPCR assay was shown to have a lower detection limit than the corresponding conventional primer-based qPCR assay (Satterfield, 2014).

2.6.3.2 Loop-mediated isothermal amplification (LAMP)

LAMP is a NAAT technique for amplification of target nucleic acid (RNA and/or DNA) under isothermal conditions (Notomi *et al*., 2000). The optimal temperature range for LAMP reaction is usually between 60ºC to 65ºC (Becherer *et al*., 2020). The process involves the use of *Bacillus stearothermophilus* (*Bst*) polymerase and four to six target-specific primer sets, which substantially improved sensitivity and specificity (Notomi *et al*., 2000). Compared to PCR, LAMP has a relatively fast turnaround time (<60 minutes) and requires a simple and low-cost operational device (Gill & Ghaemi, 2008). In addition, the resulting amplicons from the assay can be visualised using simple and relatively less-expensive colorimetric methods that are visible to the naked eye (Craw & Balachandran, 2012; Fang *et al*., 2010; Gill & Ghaemi, 2008). These field-adaptable characteristics of LAMP provide the opportunity for the application of NAAT at the POC (Craw & Balachandran, 2012).

The first LAMP assay for the detection of malaria parasites was described in 2006 for *P. falciparum* (Poon *et al*., 2006). Following this, specific-specific LAMP assays were reported for the detection of *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* using primers sets that target the 18S rRNA gene (Han *et al*., 2007). The LODs for the assays were 10 parasite copies/reaction for *P. malariae* and *P. ovale* and 100 parasite copies/reaction for *P. falciparum*

and *P. vivax* (Han *et al*., 2007). Currently, other LAMP assays have been developed for the detection of *P. malariae* and *P. ovale* using genus *Plasmodium* primer sets that target the 18S rRNA (Ngoc *et al*., 2020) and the mitochondrial DNA (Polley *et al*., 2010). Notably, the analytical performance of these LAMP assays is comparable to PCR (Picot *et al*., 2020). Despite these advantages, the routine application of LAMP for malaria diagnosis at the POC remains a challenge due to the high cost of LAMP assay reagents, particularly *Bst* polymerase (Becherer *et al*., 2020).

2.7 Biosensors

In recent years, the development of field-adaptable biosensors has received unprecedented attention due to the increase in demand for reliable, cost-effective, fast and simple POC diagnostic devices (Krampa *et al*., 2020; Mehrotra, 2016; Mohankumar *et al*., 2021). Biosensors are analytical devices that receive, amplify and process a response generated from a biological process. A biosensor has three basic components: a biorecognition receptor (e.g. antibody, antigen, DNA, or cell) which interacts with the target analyte to generate a biological response, a transducer (e.g. electrode) which receives and translates the biological response into an analytical signal and lastly a signal processing unit which process and displays the signal in a user-friendly approach (Bhalla *et al*., 2016; Grieshaber *et al*., 2008).

2.7.1 Biosensors for malaria diagnosis

Several studies have reported different types of biosensors for the diagnosis of malaria (Krampa *et al*., 2020). These biosensors employ various biorecognition receptors, including antigen, antibodies, and DNA (Krampa *et al*., 2020). Most of these malaria biosensors are immunosensors that are based on antigen-antibody interaction (Dutta, 2020; Krampa *et al*.,

2020). Due to the lack of reliable antigenic biomarkers, most of these immunosensors target the PfHRP2 and pLDH which have been reported to have several limitations, as discussed earlier in section 2.6.2 (Agaba *et al*., 2019; Simpalipan *et al*., 2018; Verma *et al*., 2018). In addition, there is currently no immunosensor for species-specific detection of *P. malariae* and *P. ovale* due to the lack of appropriate species-specific antigenic biomarkers. On the other hand, nucleic acid biomarkers are generally thought to be more advantageous than antigenic biomarkers (Cordray & Richards-Kortum, 2012). Nucleic acid-based biomarkers are relatively simple to identify and can be targeted with high selectivity (Cordray & Richards-Kortum, 2012). In addition, nucleic acid-based biosensors are relatively simple, fast, and cost-effective compared to NAATs such as PCR (Wang *et al*., 1998; Zhang *et al*., 2000). Despite these advantages, there is little advancement in the development of nucleic acid-based biosensors for the diagnosis of malaria.

2.7.2 DNA-based biosensors

A DNA-based biosensor, also called a genosensor, consists of an immobilised single-stranded DNA probe that interacts with the complementary strand to form a DNA-DNA complex at the surface of the transducer or electrode (Kavita, 2017; Wu *et al*., 2019). To achieve efficient hybridisation (sensitivity) and minimise non-specific interaction or adsorption (specificity), some major factors that must be taken into consideration include the concentration and the orientation of the immobilised single-stranded DNA probe on the electrode surface (Kanyong *et al*., 2020a). An optimal amount of probe must be carefully determined to ensure maximum biological response since suboptimal or overcrowding of the electrode surface would significantly affect the performance of the biosensor (Bizzotto *et al*., 2018). Also, proper probe orientation is necessary for efficient interaction with the target complementary DNA (Bizzotto

et al., 2018; Ulianas *et al*., 2012). Several approaches have been described for efficient probe immobilisation (Nimse *et al*., 2014; Rashid & Yusof, 2017). Examples of these strategies are biotin-avidin mediated probe immobilisation (Oberhaus *et al*., 2020), self-assembly monolayer (SAM) of thiolated probes (Ilkhani *et al*., 2013; Wang *et al*., 2014), and electro-polymerisation of probes (Rashid & Yusof, 2017).

2.7.3 Transducing mechanisms for malaria biosensors

Transducing mechanism is one of the essential factors that determine the specificity, sensitivity, cost, scalability, and portability of a biosensor (Rashid & Yusof, 2017). Various transducing mechanisms have been employed in the development of biosensors (Dutta, 2020; Krampa *et al*., 2020). Examples of these transducing mechanisms include optical approach (surface plasmon resonance, colorimetric, fluorescence, and luminescent), mass changes (quartz crystal microbalance and whispering-gallery microgravity) and electrochemical approach (amperometric, potentiometric, or impedimetric) (Nakazato, 2013; Nirschl *et al*., 2011). The transducing mechanisms that have been widely employed in the development of malaria biosensors are discussed in the subsequent sections.

2.7.3.1 Colorimetry

Colorimetric-based techniques enable the detection of target molecules based on a colour change that is visible to the naked eye (Mauriz, 2020). Some of the strategies that have been employed in the development of colorimetric biosensors include surface plasmon resonance (SPR)-mediated aggregation of nanoparticles, SPR-mediated decomposition of metallic nanostructures, enzyme-mediated catalysis, fluorescent-mediated colour change (Couture *et al*., 2013; Homola, 2008; Liu *et al*., 2018; Masson, 2020; Mauriz, 2020; Zhan *et al*., 2017).

Jeon *et al*. described genus *Plasmodium* (pLDH) colorimetric biosensor with a LOD of 74 parasites/µL using gold nanoparticles (Jeon *et al*., 2013). The presence of pLDH triggers aggregation of the gold nanoparticles that result in a visible colour change from red to blue (Jeon *et al*., 2013). Subsequently, a *P. falciparum* specific (PfLDH) colorimetric biosensor based on the intrinsic enzymatic activity of pLDH, which mediated the generation of a blue colour resulting from the reduction of 3-acetylpyridine adenine dinucleotide was reported (Dirkzwager *et al*., 2016). However, these biosensors require a large sample volume which limits their POC application. As such, the group further developed a microfluidic colorimetric biosensor using pLDH as the target biomarker (Fraser *et al*., 2018).

2.7.3.2 Quartz crystal microbalance (QCM)

QCM technique is based on the principle of mass detection (Alassi *et al*., 2017; Vashist & Vashist, 2011). QCM-based biosensors measure changes in resonance frequency in response to change in mass at the electrode surface (Qiao *et al*., 2016). The technique is a label-free method which gives it some advantages over other detection mechanisms that require labels (Lim *et al*., 2020). Potipitak *et al.* described the first QCM DNA-based biosensor for the detection of *P. falciparum* using gold electrodes (Potipitak *et al*., 2011). To reduce the diagnostic cost, the same group later developed another QCM DNA-based biosensor for the detection of *P. falciparum* and *P. vivax* using silver-based electrodes (Wangmaung *et al*., 2014). However, these QCM biosensors required initial PCR amplification of target DNA, making them less suitable for POC application due to the challenges associated with PCR-based assays (Potipitak *et al*., 2011; Wangmaung *et al*., 2014).

2.7.3.3 Surface-enhanced Raman scattering (SERS)

Raman scattering is an optical technique that uses light photons to provide information on the identity of biological analytes through interactions between the light photons and the analyte (Jones *et al*., 2019). By immobilising the analyte on metal surfaces, Raman scattering could be enhanced several million folds in a process called surface-enhanced Raman scattering (SERS) (Schlücker, 2014). SERS-based biosensors involve the use of metallic nanoparticles and demonstrate high sensitivity, robustness, and improved efficiency compared to PCR (Lane *et al*., 2015; Schlücker, 2014). Ngo *et al.* developed the first SERS-based malaria biosensor for the detection of *P. falciparum* DNA using nanorattles with LOD of 100 aM (Ngo *et al*., 2016). Subsequently, the group further developed another SERS-based biosensor using gold nanoparticles to directly detect *P. falciparum* RNA in whole blood lysate without the need for initial nucleic acid purification or target amplification (Ngo *et al*., 2018). Despite this robustness, their routine use of SERS-based biosensors at the POC has been limited by the cumbersome multiple steps, large sample volume, the expensive reagents and equipment required (Liu *et al*., 2019; D. Zhang *et al*., 2021).

2.7.3.4 Electrochemical impedance spectroscopy (EIS)

EIS is a label-free electrochemical detection technique that involves the application of a small voltage signal to measure electron transfer blockage properties occurring at the surface of an electrode (Kanyong *et al*., 2020a). EIS works by monitoring the direct transfer of free electrons between the biorecognition elements and the electrode surface without the need for an intermediate component (Nishikata, 1998; Retter & Lohse, 2010; Sharifi-Asl & Macdonald, 2014). EIS is generally simple, cost-effective, and scalable (Cheng & Toh, 2013). Several EIS-based biosensors have been reported for malaria diagnosis using various biorecognition receptors (Dutta, 2020; Krampa *et al*., 2020). Lee *et al.* described a malaria aptasensor, a

biosensor that uses aptamers as biorecognition receptors, for the detection of genus *Plasmodium* LDH (pLDH) (Lee *et al*., 2012). The aptasensor was tested on *P. falciparum* and *P. vivax* and was shown to have a limit of detection of 1.0 pM. However, the lack of specificity of this aptasensor for the detection of *Plasmodium* species limits its application for reliable diagnosis of malaria (Lee *et al*., 2012). As such, other research groups have developed *P. falciparum*-specific aptasensors using *P. falciparum* glutamate dehydrogenase (PfGDH) and *P. falciparum* LDH (PfLDH) (Paul *et al*., 2016; Singh *et al*., 2018). The LODs of the PfGDH and the PfLDH aptasensors were 0.77 pM and 0.50 fM, respectively (Paul *et al*., 2016; Singh *et al*., 2018). Currently, no EIS-based biosensor has been reported for the detection of *Plasmodium* species using parasite nucleic acid as the target analyte. As such, EIS-based biosensors were developed in this study for species-specific detection of *P. falciparum, P. malariae* and *P. ovale* genomic DNA in clinical samples.

Table 1: Summary of the analytical performance of the detection methods for *P. malariae* and *P. ovale.*

Method	Target	LOD*	Advantages	Disadvantages
Microscopy	Infected RBC	>50	Cost-effective Readily available	Lack of adequate sensitivity Lack of adequate specificity Requires well-trained microscopists
RDTs	Antigen	>100	Cost-effective Readily available Rapid Minimal expertise required	Lack of adequate sensitivity Lack of adequate specificity
PCR	DNA RNA	<10	High sensitivity High specificity	Requires high expertise Requires expensive of reagents Requires expensive equipment Not readily available at the POC
LAMP	DNA RNA	<10	High sensitivity High specificity Rapid Minimal expertise required	Not readily available Requires expensive of reagents
Biosensors	Infected RBC Antigen DNA RNA	<10	High sensitivity High specificity Rapid Minimal expertise required	Not readily available Yet to be validated at the POC

LOD = Limit of detection, RDTs = Rapid diagnostic tests, PCR = Polymerase chain reaction, LAMP = Loop-mediated isothermal amplification, RBC = Red blood cell, DNA = Deoxyribonucleic acid, RNA = Ribonucleic acid, and POC = Point-of-care. * represents number of parasites per microlitre of blood

CHAPTER THREE

3.0 Paper 1: Development of cooperative primer-based real-time PCR assays for the detection of *P. malariae* and *P. ovale*.

Specific Aim 1

To develop cooperative primer-based qPCR assays for the detection of P. malariae and P. ovale.

Hypothesis 1: qPCR assay involving cooperative primer(s) that limit primer-dimer formation would have lower detection limit than the corresponding qPCR assay involving conventional or unmodified primers.

3.1 Rationale for study aim 1

P. malariae and *P. ovale* infections are commonly detected as low-density infections (Hawadak *et al.*, 2021; Mueller *et al.*, 2007). As such, accurate detection of these low-density infections would require the use of highly sensitive and specific tools such as PCR (Hofmann *et al.*, 2015). Although PCR-based assays have improved analytical performance, the random amplification of non-specific targets such as primer-dimers remains a major limiting factor (Chou *et al.*, 1992). Of note, the probability of the formation of primer-dimers increases with decreasing concentration of target nucleic acid (Meagher *et al.*, 2018). Therefore, it has been proposed that the use of approaches that significantly limit the formation and propagation of primer-dimers would enhance the limits of detection of PCR-based assays for the detection of low-density infections (Chou *et al.*, 1992; Meagher *et al.*, 2018; Satterfield, 2014). Although several approaches that limit primer-dimer formation have been described, cooperative primers represent the first technology that simultaneously curbs primer-dimer formation and propagation (Poritz & Ririe, 2014; Satterfield, 2014). To address specific aim 1, cooperative primers were designed and used to develop qPCR assays. The qPCR assays were compared to qPCR assays involving the corresponding conventional primers. The cooperative primer-based qPCR assays were validated in a pilot study for the detection of *P. malariae* and *P. ovale* using selected clinical isolates from a larger sample pool. The results for specific aim 1 have been published in the *Journal of Molecular Diagnostics*;

https://doi.org/10.1016/j.jmoldx.2021.07.022

3.2 Abstract

The absence of reliable species-specific detection tools has hampered the accurate diagnosis of *P. malariae* and *P. ovale*. This challenge limits the introduction of appropriate measures aimed towards global malaria control and elimination. In this study, SYBR Green-based real-time quantitative PCR (qPCR) assays were developed for the detection of *P. malariae* and *P. ovale* using cooperative primers that significantly limit the formation and propagation of primer-dimers. Both the *P. malariae* and *P. ovale* cooperative primer-based qPCR assays had at least a 10-fold lower limit of detection compared to the corresponding conventional primer-based qPCR assays. More importantly, the cooperative primer-based qPCR assays were evaluated in a pilot study using 560 samples obtained from two health facilities in Ghana. The prevalence of *P. malariae* and *P. ovale* among the combined study population were 18.6% (104/560) and 5.5% (31/560), respectively. Among the positive cases, the proportions of *P. malariae* and *P. ovale* mono-infections were 3.6% (18/499) and 1.0% (5/499), respectively, with the remaining being co-infections with *P. falciparum*. The observed prevalence of both *P. malariae* and *P. ovale* were ~2-fold higher compared to the estimated national prevalence. The study demonstrates the public health importance of including detection tools with lower detection limits in routine diagnosis and surveillance of non-falciparum species.

3.3 Introduction

Accurate species-specific detection of *P. malariae* and *P. ovale* is critical for disease management and implementation of malaria control measures. However, current diagnostic tools that are readily available and most cost-effective include microscopy and antigen-based RDTs lack adequate sensitivity and specificity for the detection of these less common non-falciparum species (Gimenez *et al*., 2021; Mueller *et al*., 2007). This challenge is partly due to the morphological similarities among *Plasmodium* species and the characteristic low-density of *P. malariae and P. ovale* in clinical isolates (Erdman & Kain, 2008; Mueller *et al*., 2007; Tangpukdee *et al*., 2009). Efforts to address this gap in the diagnosis of non-falciparum species led to the development of highly sensitive and specific nucleic acid tests (NAATs), including polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) (Hofmann *et al*., 2015; Tangpukdee *et al*., 2009).

Currently, several PCR-based assays involving the use of TaqMan probes and SYBR Green have been developed for the detection of *P. malariae* and *P. ovale* (Polley *et al*., 2010; Rougemont *et al*., 2004; Rubio *et al*., 1999; Xu *et al*., 2015). Although these assays have improved sensitivity and specificity, the formation of non-specific products such as primer-dimers remains a major limiting factor, especially at low concentrations of target nucleic acid (Chou *et al*., 1992; Poritz & Ririe, 2014). Attempts to address this challenge over the years led to the development of cooperative primers, which is the first technology that simultaneously curbs primer-dimer formation and propagation (Satterfield, 2014). The cooperative primers were shown to significantly limit primer-dimer formation and propagation up to 2.5 million-fold compared to the corresponding conventional primers (Satterfield, 2014).

A cooperative primer-based real-time quantitative (qPCR) assay has been reported for the detection of *P. falciparum*, and the assay was shown to have a lower limit of detection relative to their corresponding conventional primer-based assay (Satterfield, 2014). In this study,

SYBR Green-based qPCR assays were developed for the detection of *P. malariae* and *P. ovale* using cooperative primers that target the 18S rRNA gene.

3.4 Methods

3.4.1 Primer design for cooperative primer-based qPCR assays

The 18S rRNA gene sequences of *P. falciparum* (XR_002273101.1), *P. malariae* (M54897.1), *P. ovale curtisi* (KF696371.1), *P. ovale wallikeri* (KF696364.1), and *P. vivax* (XR_003001225.1) were retrieved from the National Centre for Biotechnology Information (NCBI) database and aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, last accessed on 12-May 2021). Conserved genomic regions specific to *P. malariae* and *P. ovale* were selected for the design of the cooperative primers. Each cooperative primer consisted of a low melting temperature (Tm) short primer and a capture sequence connected by two units of hexaethylene glycol (spacer 18). Attempts to develop assays consisting of both forward and reverse cooperative primers were unsuccessful for both *P. malariae* and *P. ovale*. Since neither the forward nor the conventional reverse primers, when used alone, would produce detectable primer-dimers (Brownie *et al*., 1997), a cooperative primer was paired with a conventional primer in both the *P. malariae* and *P. ovale* assays. For *P. ovale* cooperative primer, one wobble base was introduced into capture sequence to ensure perfect complementarity to the two *P. ovale* subspecies: *P. ovale curtisi* and *P. ovale wallikeri*. The sequences of primers used in the study are shown in **Table 2**. The details of the process of primer-dimer formation, the structure of cooperative primer, and how the cooperative primer limits primer-dimer formation have been described in **Appendix A**.

Table 2: List of primers for qPCR assays.

Assay	Target	Primer code	Sequence (5'- 3')
Cooperative	*P. malariae*	PlasmoF	TTATGAGAAATCAAAGTCTTTGGGTT
		MalR3_Coop	AAAACATTCTAATATTTTAATCA[Sp18][Sp18]GGGAAAAGAACGT
Cooperative	*P. ovale*	OvaF_Coop	CTGYTCTTTGCATTCCTTAT [Sp18][Sp18]GCTTAGACAATA
		Plasmo2[a]	AACCCAAAGACTTTGATTTCTCATAA
Conventional	*P. malariae*	PlasmoF	TTATGAGAAATCAAAGTCTTTGGGTT
		MalR3	AAAACATTCTAATATTTTAATCA
Conventional	*P. ovale*	Ova_F	CTGYTCTTTGCATTCCTTAT
		Plasmo2[a]	AACCCAAAGACTTTGATTTCTCATAA
	P. falciparum	Pf_stRNA_F[b]	AAGTAGCAGGTCATCGTGGTT
		Pf_stRNA_R[b]	TTCGGCACATTCTTCCATAA

[a]Primer sequence has been previously published by Rougemont *et al.*, 2004.

[b]Primer sequence has been previously published by Heinberg *et al.*, 2013.

[SP18] represents spacer 18

3.4.2 Development of SYBR Green-based qPCR assays

The *P. malariae* and *P. ovale* SYBR Green-based qPCR assays were performed on the QuantStudio5 system (Applied Biosystems, UK). All reactions were performed in a total volume of 15 µL containing 1X Luna Universal qPCR Master Mix (New England BioLabs, UK), 250 nM of each of the cooperative and the conventional primers and 3 µL of the template

DNA. The cycling conditions for both assays consisted of 3 minutes at 95℃ followed by 45 cycles of 15 seconds at 95℃, 40 seconds at 50℃ and 40 seconds at 60℃. The specificity of the qPCR products was determined using the melting curves and on 1.5% agarose gel. The resulting gel was processed using the Amersham Imager 600 (General Electrics Healthcare Life Sciences, Chicago, USA). The performance of the cooperative primers was compared to parallel assays containing the capture sequence of each cooperative primer adopted as the conventional primer and paired with the other conventional primer that was used in the cooperative assays (**Table 2**). The assays were compared using 10-fold serially diluted MRA-179 and MRA-180 plasmids (American Type Culture Collection, Manassas, USA) for *P. malariae* and *P. ovale*, respectively, with concentrations ranging from 10^6 to 10^0 copies/µL.

3.4.3 Analytical specificity and limit of detection

The analytical specificity of the qPCR assays was determined using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, last accessed on 12-May 2021). Experimental specificity was also determined using genomic DNA of *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. The limits of detection (LODs) and the efficiency of the assays were determined using a 10-fold serial dilution of MRA-179 and MRA-180 plasmids for *P. malariae* and *P. ovale*, respectively. Each plasmid was diluted to obtain 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/µL in Tris-ethylenediaminetetraacetic acid (TE) buffer. All assays were performed in triplicates.

3.4.4 Validation of cooperative primer-based qPCR assays using clinical samples

A pilot study was conducted to assess the efficiency of the cooperative primer-based qPCR assays for the detection of *P. malariae* and *P. ovale* in clinical isolates using randomly selected samples (sample size, n = 560) from a larger pool of whole blood samples that were obtained from individuals who presented with suspected malaria at the Ewim Polyclinic in Cape Coast and the Richard Novati Catholic Hospital in Sogakope between December 2017 and December 2018. Genomic DNA was purified from 200 µL of the venous blood using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK) following instructions from the manufacturer. DNA was eluted in a total volume of 100 µL of elution buffer (Qiagen, UK). The purified genomic DNA was stored at -20^{0}C until ready for molecular analysis. To determine the diagnostic performance of the qPCR assays, microscopy analysis was performed for the identification of *Plasmodium* species. Thick and thin blood smears were prepared at the time of blood sample collection and stained with 10% Giemsa. The number of parasite-infected RBCs was determined per 500 white blood cells (WBCs). Parasite count per microlitre of blood was determined using the standard leucocyte count of 8000 leucocytes per microlitre of blood as previously described (Greenwood & Armstrong, 1991). *P. malariae* and *P. ovale* were detected using the SYBR Green cooperative primer-based qPCR assays described earlier in this study, while *P. falciparum* detection was performed using a previously described SYBR Green-based qPCR protocol with primers targeting *P. falciparum* seryl-transfer RNA synthetase gene (PF3D7_0717700) (Heinberg *et al*., 2013). The specificity of the qPCR amplicons was determined using melt curve analysis. The resulting threshold cycle (C_t) values for positive samples were used to estimate parasite copy number per microlitre using standard curves obtained from 10-fold serially diluted plasmids.

3.4.5 Statistical analyses

Data analysis was performed using IBM SPSS Statistics (version 26), GraphPad Prism (version 8.0.2), and Microsoft Excel 2016 software. Probit analysis was used to estimate the LOD of the assays at 95% confidence interval. Parasite load across three or more groups were compared using the Kruskal-Wallis test, and where differences were observed, pairwise comparisons were conducted using the Mann-Whitney U test. Statistical significance for all analyses was considered for *P*-value less than 0.05.

3.4.6 Ethical Approval

The study obtained ethical clearance from the ethics committees of the Ghana Health Service (GHSERC005/12/17), the Noguchi Memorial Institute for Medical Research, University of Ghana (NMIMR-IRB CPN 077/17-18) and the Kintampo Health Research Centre (KHRCIEC/2018-10). Written informed consent was obtained from all participants and/or from parents or guardians of participants.

3.5 Results

3.5.1 Analytical sensitivity, specificity and limit of detection

SYBR Green-based qPCR assays for the detection of *P. malariae* and *P. ovale* were developed using cooperative primers. Analysis of amplicons using melt curves (**Figures 2A and 2B**) and agarose gel electrophoresis (**Figures 2C and 2D**) showed that both assays were specific to the selected 18S rRNA genomic region with no cross-reactivity to other *Plasmodium* species. The melting temperature (Tm) of the assays were 85.60 ± 0.46 ^{o}C and 79.44 ± 0.17 ^{o}C for *P. malariae* and *P. ovale*, respectively (**Table 3**). Using 10-fold serially diluted plasmids, the limits of detection estimated at 95% confidence level for the *P. malariae* and *P. ovale* assays were 1.0 plasmid copy/µL (CI_{95} [0.94 – 1.06]) and 1.0 plasmid copy/µL (CI_{95} [0.96 – 1.04]), respectively (**Figure 2E**). The amplification efficiencies of the assays were 68.1 % and 76.9 % for *P. malariae* and *P. ovale*, respectively **(Table 3)**.

3.5.2 Comparison of the cooperative and conventional primers

The analytical performance of the cooperative primers was compared to the corresponding conventional primers. The C_t-values observed for the conventional primer-based qPCR assays were relatively lower compared to the corresponding cooperative primer-based qPCR assays (**Table 4**). Despite this observation, the cooperative primer-based qPCR assays detected as low as 1.0 copy/µL, whereas the conventional primer-based qPCR assays had limit of detection of 10.0 copies/µL for both the *P. malariae* and *P. ovale* assays (**Table 4**). Separation of the resulting qPCR amplicons on a 1.5% agarose gel showed primer-dimers as the concentration of *P. malariae* and *P. ovale* decreased for the conventional primer-based qPCR assays (**Figures 3A and 3C)**. On the contrary, no detectable primer-dimers were generated for both the *P.*

malariae and *P. ovale* cooperative primer-based qPCR assays even at the lowest concentration of 1.0 copy/µL (**Figures 3B and 3D**). Taken together, the data suggest that the cooperative primer-based qPCR assays have 10-fold lower LOD compared to using conventional primer-based qPCR assays.

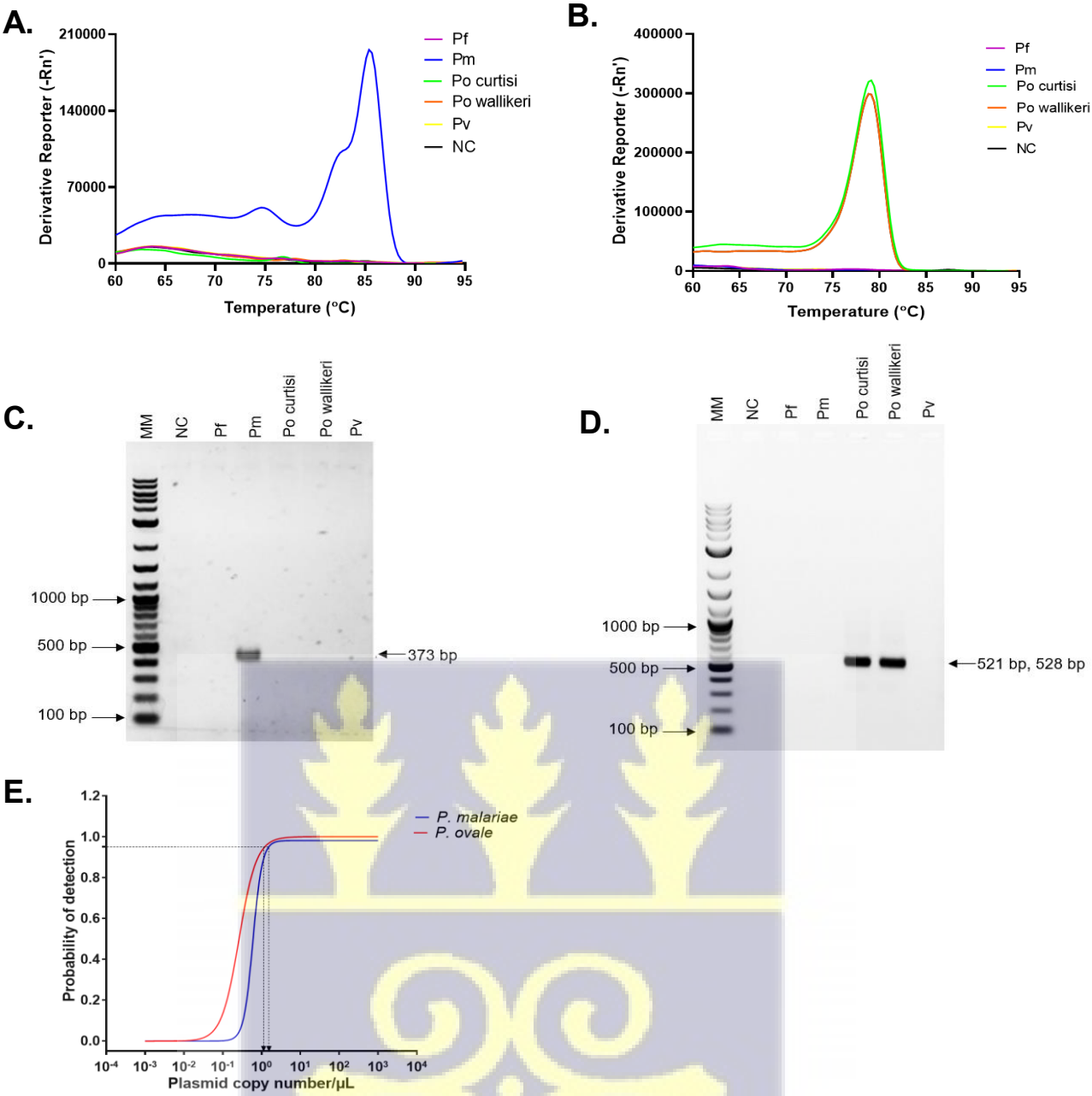

Figure 2: The specificity and limits of detection for cooperative primer-based qPCR assays.

Assays were performed using *P. falciparum* (Pf), *P. malariae* (Pm), *P. ovale curtisi* (*Po curtisi*), *P. ovale wallikeri* (*Po wallikeri*) and *P. vivax* (Pv) genomic DNA. **(A)** Melt curve for *P. malariae* (blue) **(B)** Melt curves for *P. ovale curtisi* (green) and *P. ovale wallikeri* (orange) assays. Other colours represent the *P. falciparum, P. vivax* and non-template negative control (NC). **(C)** Separation of the resulting *P. malariae* qPCR amplicons on 1.5% agarose gel. **(D)** Separation of the resulting qPCR amplicons for *P. ovale* on 1.5% agarose gel. Expected amplicon sizes for *P. ovale curtisi* and *P. ovale wallikeri* were 528 base-pair (bp) and 521 bp, respectively. Molecular weight marker (MM) shown in base pairs (bp) **(E)** The limits of detection for *P. malariae* (blue) and *P. ovale* (red) assays were determined using a 10-fold serial dilution of plasmids. The probability of detection was plotted against the plasmid copy numbers/µL of the DNA.

Table 3: The efficiencies of cooperative primer-based qPCR assays.

Assay	Slope	Intercept	R^2	Efficiency (%)	Amplicon length (bp)	Melting temperature[a] (ºC)
P. malariae	-4.4	39.10	0.99	68.1	373	85.60 ± 0.46
P. ovale	-4.0	38.73	0.99	76.9	528[b], 521[c]	79.44 ± 0.17

[a] Mean melting temperature for technical replicates

[b] Expected qPCR amplicon base-pair (bp) length for *P. ovale curtisi*

[c] Expected qPCR amplicon base-pair (bp) length for *P. ovale wallikeri*

Table 4: Comparison of C_t-values for cooperative and conventional primer-based assays.

Plasmid copies/µL	*P. malariae*		*P. ovale*	
	Conventional	Cooperative	Conventional	Cooperative
10^6	9.83 ± 0.07	11.15 ± 0.15	12.94 ± 0.01	13.70 ± 0.04
10^5	13.57 ± 0.18	17.53 ± 0.28	16.60 ± 0.21	17.38 ± 0.07
10^4	16.96 ± 0.39	21.90 ± 0.36	21.46 ± 0.11	22.65 ± 0.05
10^3	21.22 ± 0.38	27.22 ± 0.25	26.11 ± 0.15	27.46 ± 0.13
10^2	25.31 ± 0.22	33.00 ± 0.45	28.15 ± 0.13	29.65 ± 0.14
10^1	27.35 ± 0.09	38.28 ± 0.36	32.75 ± 0.16	34.20 ± 0.29
10^0	Negative	41.94 ± 0.54	Negative	38.77 ± 2.15

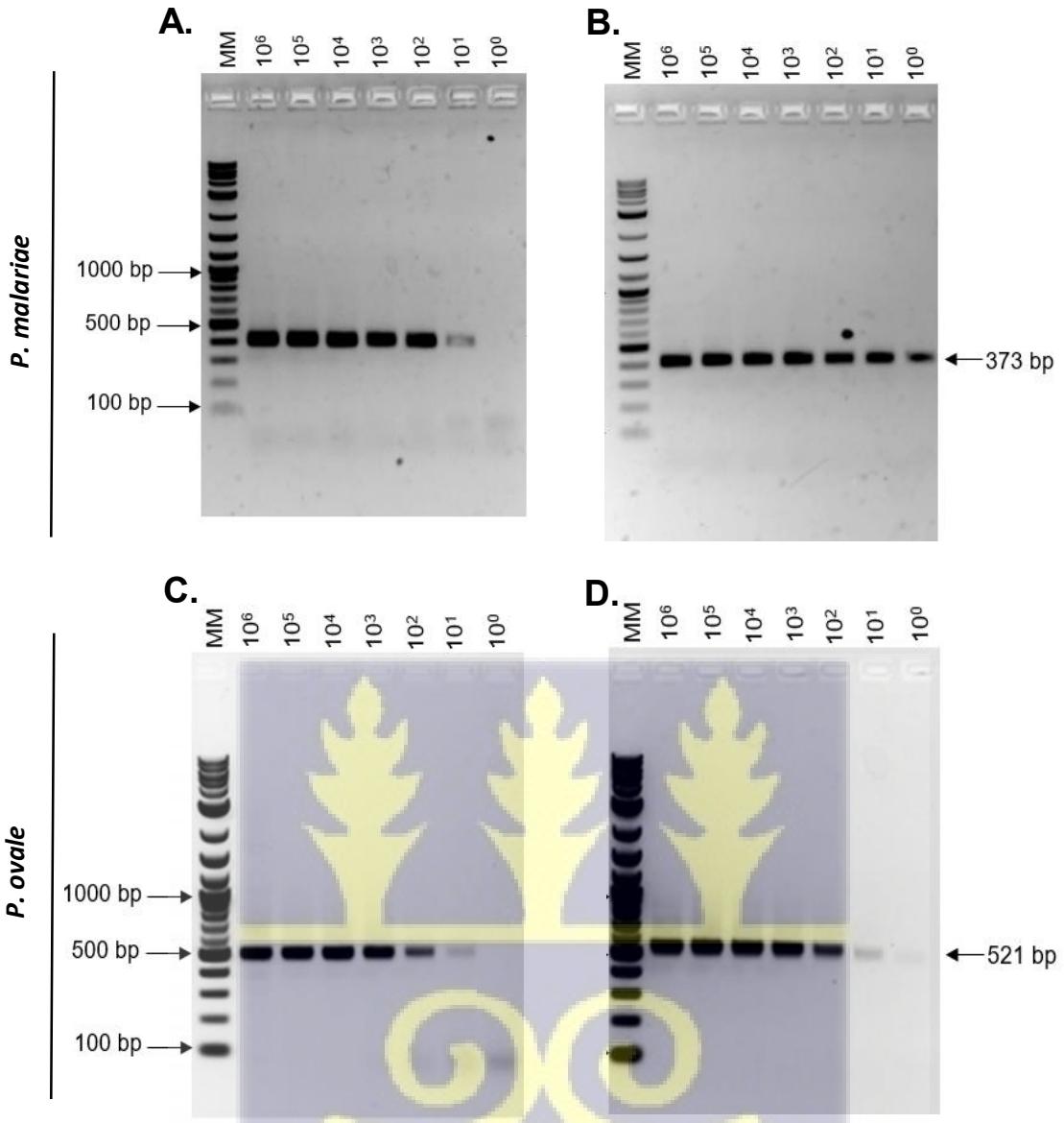

Figure 3: Comparison of cooperative and conventional primer-based qPCR assays.

Assays were performed using serially diluted plasmids for *P. malariae* conventional primer-based assay (**A**), *P. malariae* cooperative primer-based assay (**B**), *P. ovale* conventional primer-based assay (**C**) and *P. ovale* cooperative primer-based assay (**D**). The qPCR amplicons were separated on 1.5% agarose gel. Molecular weight marker (MM) shown in base pairs (bp).

3.5.3 Prevalence of *P. falciparum*, *P. malariae* and *P. ovale* among the study participants

The prevalence of *P. falciparum*, *P. malariae* and *P. ovale* among the study participants (n = 560) was assessed by microscopy and SYBR Green-based qPCR assays. The overall prevalence of *Plasmodium* species infection by qPCR and microscopy were 89.1% (499/560) and 67.0% (375/560), respectively (**Figure 4A**). For species identification by microscopy, the prevalence of *P. falciparum*, *P. malariae* and *P. ovale* for the combined study population were 66.4% (372/560), 2.5% (14/560) and 1.1% (6/560), respectively (**Figure 4B**). As expected, higher prevalence was observed using qPCR for each of the three *Plasmodium* species than microscopy. The prevalence of *P. falciparum*, *P. malariae* and *P. ovale* by qPCR were 85.5% (479/560), 18.6% (104/560) and 5.5% (31/560), respectively (**Figure 4B**). A total of 3.2% (18/560) of the participants were negative by both microscopy and qPCR for all the three *Plasmodium* species. For discrepancies between microscopy and qPCR, 7.5% (42/560) of the microscopy-positive participants were qPCR negative for the three *Plasmodium* species. Also, 29.8% (167/560) of the qPCR positives were undetected by microscopy. Among the qPCR-positive participants that were undetected by microscopy, 76.6% (128/167), 2.4% (4/167) and 1.8% (3/167) were found to harbour mono-infections for *P. falciparum*, *P. malariae* and *P. ovale*, respectively, while the remaining 19.2% (32/167) harboured mixed infections of two or three *Plasmodium* species.

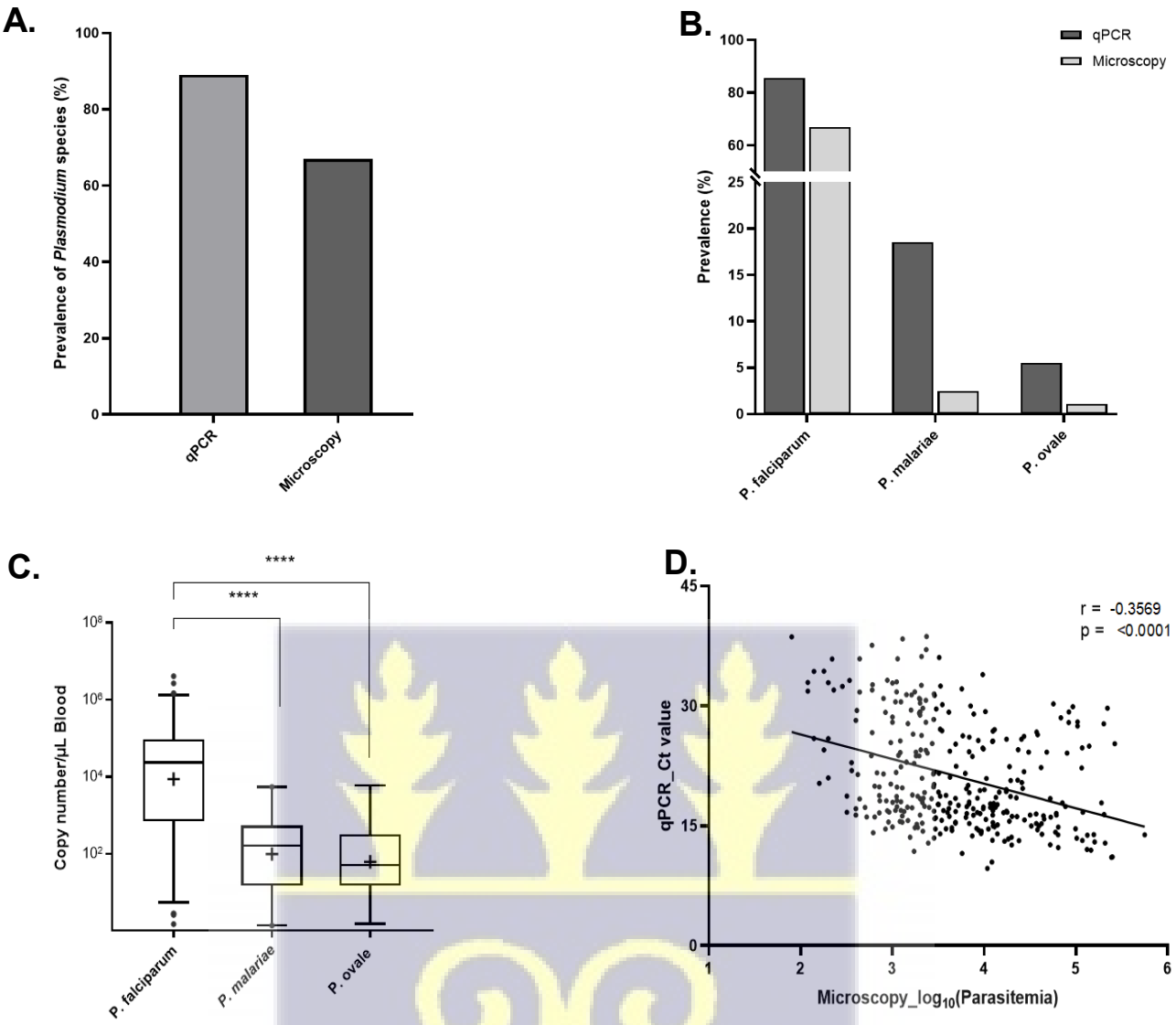

Figure 4: Prevalence of *Plasmodium* species among study participants.

(A) The overall prevalence of *Plasmodium* species determined by microscopy and qPCR **(B)** The prevalence of *P. falciparum*, *P. malariae* and *P. ovale* determined by SYBR Green-based qPCR assays and microscopy among the combined study population **(C)** The overall parasite load for *P. falciparum* (n = 479), *P. malariae* (n = 104) and *P. ovale* (n = 31) positive samples. Data have been presented as box plot where the boxes represent the interquartile range, the line through the box is the median and the whiskers indicate the 1st and the 99th percentiles. The geometric mean of the parasite load is denoted by the plus symbol (+), while the individual dots represent outliers. Parasite copy numbers were compared using the Mann-Whitney U test**.** Statistical significance has been represented as *P < 0.05, ***P < 0.001, ****P < 0.0001 **(D)** Correlation of parasite load determined by microscopy and qPCR C_t-values. r represents Pearson's correlation coefficient, and p represents probability value.

3.5.4 Quantification of parasite copy number

The copy numbers of *P. falciparum*, *P. malariae* and *P. ovale* were estimated for the positive clinical samples based on the qPCR C_t-values. The median parasite load of *P. falciparum* was significantly higher than those for *P. malariae* (**P < 0.0001**) and *P. ovale* (**P < 0.0001**) (**Figure 4C)**. However, there was no significant difference between *P. malariae* and *P. ovale* parasite loads (**P = 0.16, Figure 4C**). Lastly, the qPCR C_t-values were correlated with parasitaemia as determined by microscopy (**Figure 4D**). As expected, there was a significant negative correlation between the qPCR C_t-values and the $\log_{10}$-transformed parasitaemia (**r = - 0.36, P < 0.001**); however, the association was not very strong.

3.6 Discussion and conclusion

Due to the limited geographical distribution and marginal contribution of *P. malariae* and *P. ovale* towards the global malaria burden, these non-falciparum species have not received much attention (Doderer-Lang *et al*., 2014). However, it is generally thought that the reported prevalence of these non-falciparum species has been largely underestimated due to the lack of reliable diagnostic tools (Farcas *et al*., 2003; Nino *et al*., 2016). As such, there is the need for reliable diagnostic tools to accurately assess the prevalence and the burden of *P. malariae* and *P. ovale*. In this study, cooperative primers were used to develop qPCR assays for the detection of *P. malariae* and *P. ovale* in clinical isolates.

One major obstacle that limits the specificity and the sensitivity of NAATs is the formation and propagation of non-specific products such as primer-dimers which result in false negatives or false positives (Meagher *et al*., 2018; Satterfield, 2014). Although several technologies have been described to mitigate this challenge, cooperative primers were the first technology that was shown to simultaneously inhibit the formation and propagation of primer-dimers up to 2.5 million-fold compared to conventional primers (Satterfield, 2014). This study describes the first report on the application of cooperative primers for the detection of *P. malariae* and *P. ovale*. The data presented here suggest that the cooperative primers had relatively higher C_t-values than their corresponding conventional primers for a given concentration of target DNA. A possible explanation is that there is an initial binding of the cooperative sequence before the binding of the short low-Tm primer to its complementary sequence (Satterfield, 2014), and this additional time may be a lagging phase that accounts for the differences in the C_t-values between the cooperative and the conventional primers. Notwithstanding this observation, the results show that the cooperative primer-based qPCR assays have at least a 10-fold lower limit of detection compared to their corresponding conventional primer-based qPCR assays. This result, along with previous report (Satterfield, 2014) are in line with our hypothesis that qPCR

assay involving cooperative primer(s) that limit primer-dimer formation have lower detection limit than the corresponding qPCR assays involving conventional or unmodified primers. The lower limit of detection of the cooperative primer-based assays may be explained by the ability of the cooperative primers to significantly limit primer-dimer formation (Bacich *et al*., 2011; Satterfield, 2014).

In Ghana, *P. falciparum*, *P. malariae* and *P. ovale* are the three *Plasmodium* species that have been implicated in clinical malaria (Ghana MICS, 2011). The estimated national prevalence of *P. falciparum*, *P. malariae* and *P. ovale* are 90% - 98%, <10%, and <2%, respectively (Ghana MICS, 2011). However, different studies across various sites in Ghana have reported varying prevalence rates for the three *Plasmodium* species (Amoah *et al*., 2019; Dinko *et al*., 2013; Owusu *et al*., 2017). The data presented here by microscopy show that the prevalence of *P. malariae* and *P. ovale* are comparable to the estimated national prevalence. Using qPCR analysis, the prevalence of *P. falciparum*, *P. malariae* and *P. ovale* among the study population were 85.5%, 18.5% and 5.5%, respectively. These prevalence rates of *P. malariae* and *P. ovale* are comparable to those in a previous report in Ghana (Dinko *et al*., 2013) but are about 2-fold higher than the reported national prevalence and also higher than the rates in studies conducted elsewhere in the country (Amoah *et al*., 2019; Owusu *et al*., 2017). The higher prevalence reported in this study may be due to the lower limits of detection of the cooperative primer-based qPCR assays, even though these studies involve different study populations and communities. An undetected population harbouring non-falciparum species is of great concern since these individuals potentially serve as parasite reservoirs for sustained and long-term transmission of *P. malariae* and *P. ovale*.

P. malariae and *P. ovale* infections are usually detected as coinfections with *P. falciparum* (Kasehagen *et al*., 2006; Mehlotra *et al*., 2000; Mueller *et al*., 2007). In areas of high transmission, *P. falciparum* has been reported to suppress the prevalence and the density of

non-falciparum species (Boudin *et al*., 1991; Molineaux *et al*., 1980; Mueller *et al*., 2007). Consistent with previous reports, the results show that the parasite density of both *P. malariae* and *P. ovale* parasites in all the mixed infection cases were lower than *P. falciparum*. The low density of non-falciparum species in cases of mixed infection with dominant *P. falciparum* is likely to result in misdiagnosis and affect the recommendation of the most appropriate antimalarial drug (Barber *et al.*, 2013a). However, it has been argued that since artemisinin combination therapies (ACTs) are generally effective for the treatment of uncomplicated falciparum and non-falciparum malaria (World Health Organisation, 2015), such misdiagnosis would be of less importance for antimalarial treatment. Nevertheless, with recent reports of increasing prevalence of non-falciparum species despite ACT treatment, achieving accurate species-specific detection is necessary (Betson *et al*., 2014, 2018; Calleri *et al*., 2013; Rutledge *et al*., 2017; Smith *et al*., 2011).

In summary, the results presented indicate at least a 2-fold higher prevalence of *P. malariae* and *P. ovale* among study participants compared to the estimated national prevalence in Ghana. This finding underscores the need for the deployment of detection tools with lower detection limits to accurately assess the burden of non-falciparum species. The turnaround time of the cooperative primer-based assays is comparable to conventional qPCR assays. It is important to highlight that the cost of cooperative primers is relatively higher than their corresponding conventional primers. Notwithstanding the high cost, the deployment of such detection tools will be helpful for reliable diagnosis and accurate surveillance of non-falciparum species in a holistic approach towards malaria elimination.

CHAPTER FOUR

4.0 Paper 2: The distribution of non-falciparum *Plasmodium* species in Ghana and their association with severe malaria.

Specific Aim 2

a) *To determine the prevalence of P. malariae and P. ovale in different malaria transmission settings in Ghana.*

b) *To determine the associations between P. malariae and P. ovale infections and haematological parameters.*

Hypothesis 2: Mixed infection of two or more Plasmodium species is associated with altered haematological parameters and severe malarial anaemia.

4.1 Rationale for Specific Aim 2

Plasmodium species infection is well known to induce alteration in haematological indices such as haemoglobin levels, white blood cell (WBC) counts, and platelet counts (Price *et al*., 2001; Wickramasinghe & Abdalla, 2000). These changes have been reported as essential indicators of malaria infection and severity (Purbiya *et al*., 2018; Zhang *et al*., 2015). In addition, mixed infections of *Plasmodium* species have been shown to significantly increase the risk of developing severe anaemia compared to mono-infections of *Plasmodium* species (Douglas *et al*., 2013). In sub-Saharan Africa, submicroscopic *P. malariae* and *P. ovale* are usually detected as mixed infections with the dominant *P. falciparum* (Mueller *et al*., 2007). Despite this knowledge, most of these association studies have mainly focused on the dominant *P. falciparum*, with little known about how *P. malariae* and *P. ovale* modulate haematological indices (Douglas *et al*., 2013). This gap is partly due to the absence of reliable tools for accurate detection of *P. malariae* and *P. ovale*. In Specific Aim 1, the new cooperative primer-based qPCR assays were developed and validated using a small sample size in a pilot study. Therefore, Specific Aim 2 was performed using the cooperative primer-based qPCR assays to determine the prevalence rates of *P. malariae* and *P. ovale* among a large cohort of symptomatic and asymptomatic individuals from three different malaria transmission settings in Ghana. The effects of *P. malariae* and *P. ovale* mono and mixed infections on six haematological parameters (haemoglobin levels, haematocrit, WBC counts, lymphocyte counts, monocyte counts, granulocyte counts and platelet counts) were assessed.

4.2 Abstract

Alterations in haematological indices following infection of *Plasmodium* parasites have been reported as a major contributing factor to malaria severity. However, most of these studies are centred on the dominant *P. falciparum* and *P. vivax* parasites, with little known about how *P. malariae* and *P. ovale* alter haematological indices. This is partly due to the limited prevalence of non-falciparum species and the lack of reliable detection tools. Using the SYBR Green-based real-time quantitative PCR (qPCR) assays described in our previous study (**Chapter 3**), we first determined the distribution of *P. falciparum*, *P. malariae* and *P. ovale* among symptomatic (n = 1,233) and asymptomatic (n = 1,307) participants from three malaria transmission settings in Ghana. Following this, the associations between *P. falciparum*, *P. malariae* and *P. ovale* infections among symptomatic participants and haematological indices were assessed. The prevalence of *P. falciparum*, *P. malariae* and *P. ovale* among symptomatic participants were 88.5% (1091/1233), 15.3% (189/1233) and 5.2% (64/1233), respectively. The distribution of *P. falciparum* and *P. malariae* were significantly higher among symptomatic participants than asymptomatic participants. However, *P. ovale* distribution was comparable between the symptomatic and the asymptomatic participants. Generally, participants with mono and mixed infections of *P. falciparum* and *P. malariae* had reduced levels of haemoglobin, platelets, haematocrit, monocytes, granulocytes and lymphocytes compared to participants without *Plasmodium* infection. On the contrary, *P. ovale* infection did not significantly affect these haematological indices. Notably, participants harbouring mixed infections of *P. falciparum* with either *P. malariae* or *P. ovale* had the greatest risk of developing mild or moderate anaemia. These findings highlight the public health importance of integrating control measures that target both falciparum and non-falciparum *Plasmodium* species into malaria control programs.

4.3 Introduction

Plasmodium species infection is well known to induce alteration in haematological indices such as haemoglobin, white blood cells (WBCs) and platelets (Price *et al*., 2001; Wickramasinghe & Abdalla, 2000). These changes have been reported as important indicators of malaria infection and severity (Purbiya *et al*., 2018; Zhang *et al*., 2015). Although several factors, including haemoglobinopathy, demographic characteristics, and malaria immunity influence these haematological parameters, mono and mixed infections of *Plasmodium* species are known to significantly alter haematological profile (Douglas *et al*., 2013; Price *et al*., 2001; Wickramasinghe & Abdalla, 2000). Nevertheless, most of these association studies have focused on *P. falciparum* and *P. vivax*, with little known about how *P. malariae* and *P. ovale* infections affect haematological indices.

Infections of *Plasmodium* species are generally characterised by low-to-normal levels of WBCs (McKenzie *et al*., 2005; Tangpukdee *et al*., 2008). This phenomenon is commonly thought to result from the localisation of leukocytes away from peripheral blood other than WBC depletion (Beale *et al*., 1972; Kelton *et al*., 1983; Levin *et al*., 1973). In one study, a significant difference in WBC counts between *P. falciparum* and *P. vivax* infected patients was reported (Richards *et al*., 1998). However, other studies which did not find a significant difference between *P. falciparum* and *P. vivax* infected patients (McKenzie *et al*., 2005; Perrin *et al*., 1982; Rojanasthien *et al*., 1992). Also, children with malaria have been reported to have a higher WBC count than healthy controls from the community (Ladhani *et al*., 2002). On the contrary, another study observed that individuals with patent parasite loads had lower WBCs compared to healthy controls (McKenzie *et al*., 2005). To date, the effect of *Plasmodium* species infections on WBCs remains poorly understood.

Some evidence suggests that platelets play a significant protective role against *Plasmodium* infection (Cox & McConkey, 2010; Kho *et al*., 2018; Wassmer *et al*., 2008). The proposed

mechanisms for this protective role include inhibition of *P. falciparum* invasion of erythrocytes (Jongruamklang *et al*., 2021) and direct killing of *falciparum*-infected RBCs (Kho *et al*., 2018). However, other studies found no evidence of erythrocyte invasion or direct killing of *falciparum*-infected RBCs (Gramaglia *et al*., 2017). Several cases of thrombocytopenia, a condition characterised by low platelet counts, have been observed among non-immune (Eriksson *et al*., 1989; Robinson *et al*., 2001) and semi-immune individuals with *Plasmodium* species infection (Lee *et al*., 1997; Pukrittayakamee *et al*., 1989). Although the mechanism underlying the reduction in platelet count remains unclear, it has been suggested that immune-mediated destruction of platelets (Kelton *et al*., 1983; Mohanty *et al*., 1988), consumption of platelets during coagulation (Pukrittayakamee *et al*., 1989) and removal of platelets by the reticuloendothelial system (Beale *et al*., 1972; Levin *et al*., 1973) may account for thrombocytopenia (Fajardo, 1974). In Africa, platelets have been shown to play an important role in malaria severity (Gérardin *et al*., 2002). In one study, platelet sequestration in the cerebral micro-vessels was observed among children who died from cerebral malaria, which suggest the role of platelets in malaria prognosis (Pongponratn *et al*., 1985).

In sub-Saharan Africa, *P. malariae* and *P. ovale* infections are usually detected as co-infection with *P. falciparum* (Hawadak *et al*., 2021; Mueller *et al*., 2007). Of note, mixed infections of *Plasmodium* species have been shown to significantly increase the risk of developing severe anaemia compared to mono infections of *Plasmodium* species (Douglas *et al*., 2013). However, there is limited data on the extent to which mono and mixed infections of *P. malariae* and *P. ovale* contribute to severe malaria. Therefore, in this study, we determined the prevalence of *P. falciparum*, *P. malariae* and *P. ovale* among study participants and further assessed their association with haematological indices.

4.4 Methods

4.4.1 Study sites and design

The study involves a cross-sectional hospital-based and community-based recruitment of symptomatic and asymptomatic participants from three selected communities in Ghana. Study participants were recruited from communities in Cape Coast, Sogakope and Kintampo North (**Figure 5**). Cape Coast is located in the Central Region of Ghana with all-year malaria transmission; however, malaria peaks during the June-July rainy season (Ayanful-Torgby *et al*., 2018). Sogakope is located in the South-Tongu district in the Volta region of Ghana, where malaria transmission is usually all year but peaks during the June-July rainy season (Ayeh-Kumi *et al*., 2016; Mba & Aboh, 2007). Kintampo North Municipality is located within the forest savannah in the Bono East region of Ghana. Malaria transmission in Kintampo is all year with seasonal peaks between April and October (Dery *et al*., 2010). These three communities span different ecological regions with varying malaria transmission intensities. Both Cape Coast and Sogakope are considered meso-endemic settings for malaria transmission, while Kintampo is a hyper-endemic setting.

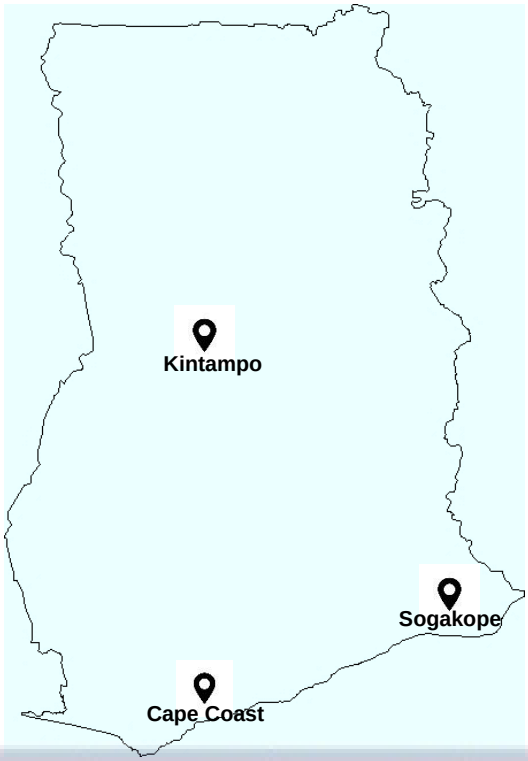

Figure 5: Map of Ghana showing study sites for sample collection.

Cape Coast and Sogakope are located in the coastal region, while Kintampo is situated in the middle belt of Ghana.

4.4.2 Sample size consideration

Sample size projections were based on the prevalence of *P. falciparum* (85.5%), *P. malariae (*18.6%) and *P. ovale* (5.5%) from our previous study (**Chapter 3**). Generally, a correlation coefficient of 0.3 with a power of 80% and an alpha (α) of 0.05 level is considered sizable for correlation analysis (Bujang & Baharum, 2016; Cohen & Maydeu-Olivares, 1992). To achieve this, minimum cases of 84 for each of the three *Plasmodium* species were required. Based on the estimated prevalence, a total of 1530 participants were required to allow a sizable meta-analysis encompassing all the participants.

4.4.3 Sample collection

For hospital-based recruitment, a total of 1,233 individuals presenting with suspected malaria at the Ewim Polyclinic in Cape Coast (n = 244), the Richard Novati Catholic Hospital in Sogakope (n = 532) and the Kintampo Municipal Hospital (n = 457) were recruited between the period of July 2018 to December 2019. A volume of 5.0 mL venous blood was collected from each participant for haematological analysis and molecular assays. In the community-based enrolment, a total of 1,307 individuals living in communities within Cape Coast (n = 498), Sogakope (n = 306) and Kintampo (n = 503) with no symptoms of any disease were recruited between July 2018 and June 2019. A volume of 5.0 mL venous blood was collected from participants for molecular assays.

4.4.4 Determination of haematological parameters

The venous blood samples obtained from symptomatic participants in the hospital-based study were used for haematological analysis using an automated haematology analyser. The haematological indices that were analysed include haemoglobin levels, haematocrit, WBCs, lymphocytes, monocytes, granulocytes and platelet indices were determined.

4.4.5 Detection of *P. falciparum, P. malariae* and *P. ovale* in clinical isolates

A volume of 200 µL of the venous blood was used for genomic DNA purification using the QIAamp DNA Mini Kit (Qiagen, Manchester, UK) following instructions from the manufacturer. DNA was eluted in a total volume of 100 µL elution buffer and stored at -20^{0}C until ready for molecular analysis. *P. falciparum*, *P. malariae* and *P. ovale* were detected using SYBR Green-based assays initially described in our previous study (**Chapter 3**). The *P. falciparum* assay involved conventional primers that target the *P. falciparum* seryl-transfer

RNA synthetase gene, while *P. malariae* and *P. ovale* assays involved cooperative primers that target the 18S rRNA gene. The details of the assay development procedures have been described in our previous study (**Chapter 3**). Briefly, all the qPCR assays were performed on the QuantStudio5 system (Applied Biosystems) in a total reaction volume of 15 µL containing 1X Luna Universal qPCR Master Mix (New England BioLabs, UK), 250 nM of each primer and 3 µL of the template DNA. The cycling conditions for the assays consisted of 3 minutes at 95℃ followed by 45 cycles of 15 seconds at 95℃, 40 seconds at 50℃ and 40 seconds at 60℃. The specificity of the resulting qPCR products was determined using the melting curve analysis. The resulting threshold cycle (C_t) values for positive samples were used to estimate parasite copy number per microlitre using standard curves obtained from 10-fold serially diluted plasmids as initially described (**Chapter 3**).

4.4.6 Statistical analyses

The IBM SPSS Statistics (version 26), GraphPad Prism (version 8.0.2) and Microsoft Excel (2016) were used to analyse the data. Comparisons across three or more groups were performed using the Kruskal-Wallis test, and where differences were observed, pairwise comparison was conducted using the Mann-Whitney U test. Statistical significance for the frequencies of *Plasmodium* species were determined using the Chi-Square test. Pearson's correlation test and multivariate linear and logistic regression analyses were used to examine the associations between variables. All variables were first assessed for normality using the Kolmogorov-Smirnov test before correlation analyses, and variables with significant departure from normality were $\log_{10}$-transformed. Statistical significance for all analyses was considered for *P*-values < 0.05.

4.4.7 Ethical consideration

The study protocol was approved by the ethics committees of the Ghana Health Service (GHSERC005/12/17), the Noguchi Memorial Institute for Medical Research, University of Ghana (NMIMR-IRB CPN 077/17-18) and the Kintampo Health Research Centre (KHRCIEC/2018-10). All participants and/or guardians of participants gave written informed consent before recruitment.

4.5 Results

4.5.1 Distribution of *Plasmodium* species among symptomatic and asymptomatic study participants

In our previous study (**Chapter 3**), qPCR assays were developed and evaluated in a pilot study. In this study, the qPCR assays were used to determine the distribution of *P. falciparum* (*Pf*), *P. malariae* (*Pm*) and *P. ovale* (*Po*) among symptomatic individuals (n = 1,233) presenting to the hospital with suspected malaria and community-based asymptomatic individuals (n = 1,307) from three selected communities in Ghana (**Table 5**).

Table 5: Demographic characteristics of the study participants.

	SYMPTOMATIC			ASYMPTOMATIC		
Study site	Cape Coast	Sogakope	Kintampo	Cape Coast	Sogakope	Kintampo
Sample size, n	244	532	457	498	306	503
Gender (n, %)						
Female	136 (55.7)	299 (65.2)	212 (46.4)	266 (53.4)	154 (50.3)	287 (57.1)
Male	108 (44.3)	233 (43.8)	245 (53.6)	232 (46.6)	152 (49.7)	216 (42.9)
Age, years (n, %)						
≤ 5	62 (25.4)	129 (24.2)	355 (77.7)	16 (3.2)	68 (22.2)	113 (22.5)
6 - 10	67 (27.5)	76 (14.3)	73 (16.0)	127 (25.5)	70 (22.9)	138 (27.4)
11 - 20	58 (23.8)	133 (25.0)	29 (6.3)	155 (31.1)	76 (24.8)	105 (20.9)
21 - 40	32 (13.1)	117 (22.0)	0 (0.0)	65 (13.1)	49 (16.0)	73 (14.5)
> 40	25 (10.2)	77 (14.5)	0 (0.0)	135 (27.1)	43 (14.1)	74 (14.7)

The overall prevalence rates of *P. falciparum*, *P. malariae* and *P. ovale* among the symptomatic study participants were 88.5% (1091/1233), 15.3% (189/1233) and 5.2% (64/1233) (**Figure 6A**). Among the asymptomatic participants, the prevalence rates of *P. falciparum*, *P. malariae* and *P. ovale* were 42.0% (549/1307), 11.5% (150/1307) and 4.5% (59/1307) (**Figure 6A**). The frequency distribution of *P. falciparum* (Chi-square, $\chi^2 = 599.10$, $P < 0.0001$) and *P. malariae* ($\chi^2 = 8.14$, $P = 0.004$) were significantly higher among the symptomatic participants than asymptomatic participants. However, the difference in the frequency of *P. ovale* between the symptomatic and asymptomatic participants was not statistically significant ($\chi^2 = 0.63$, $P = 0.427$).

Among the *Plasmodium*-positive symptomatic participants, the proportion of *P. falciparum* mono-infection in Cape Coast was comparable to that in Sogakope ($\chi^2 = 0.004$, $P = 0.947$) but significantly lower than that in Kintampo ($\chi^2 = 10.98$, $P < 0.001$) (**Figure 6B**). Both *malariae* and *P. ovale* mono infections were comparable across the three study sites ($P > 0.05$ for all comparisons). For mixed infections analysis, *Pf/Pm* mixed infections were significantly lower in Kintampo compared to that in Cape Coast ($\chi^2 = 8.14$, $P = 0.004$) and Sogakope ($\chi^2 = 27.88$, $P < 0.0001$). However, *Pf/Po* mixed infection was comparable across the three study sites ($\chi^2 = 1.38$, $P = 0.50$).

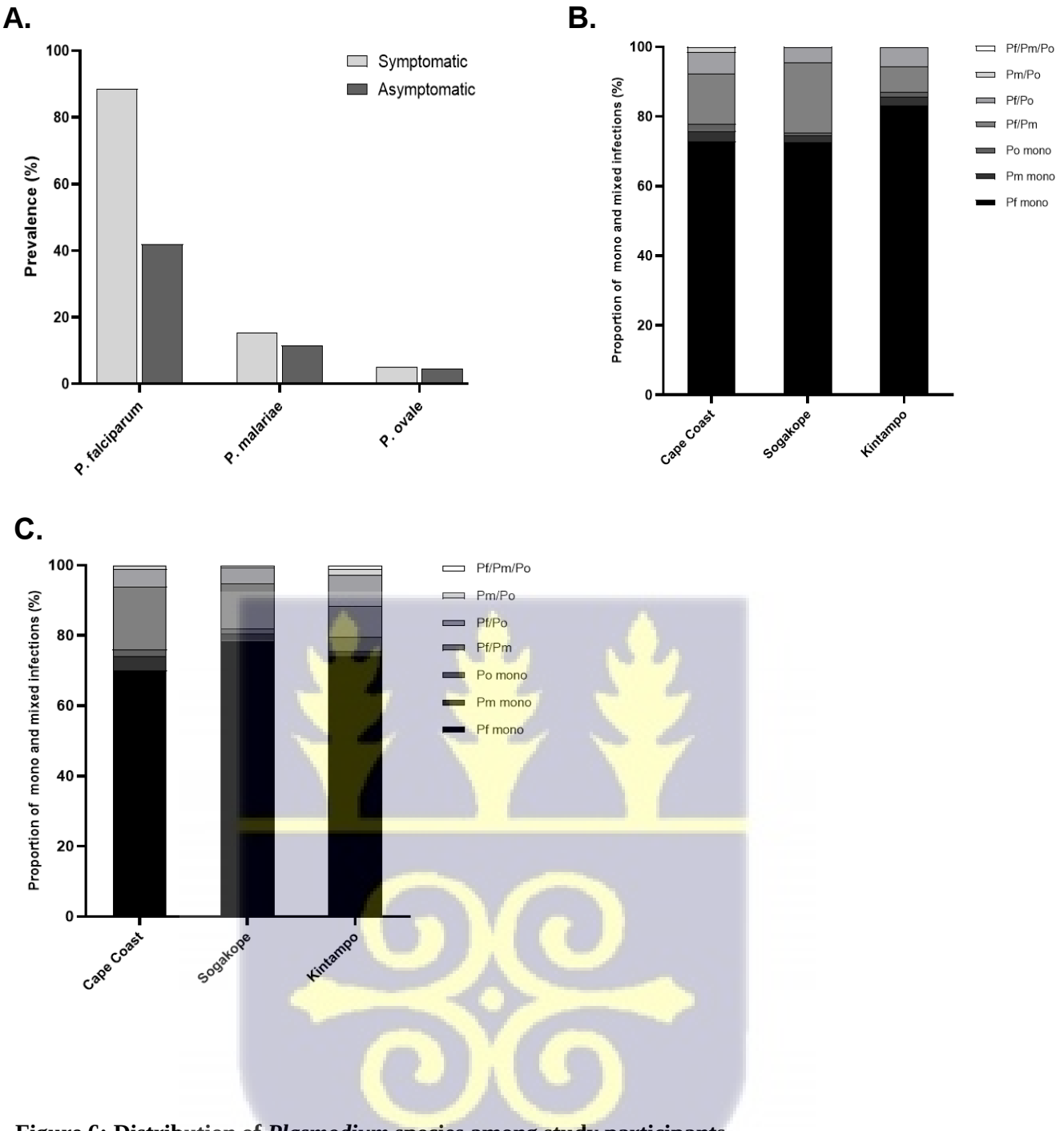

Figure 6: Distribution of *Plasmodium* species among study participants.

(A) The overall prevalence of *P. falciparum*, *P. malariae* and *P. ovale* among symptomatic (n = 1,233) and asymptomatic (n = 1,307) participants. **(B)** The proportions of mono and mixed infections of *Plasmodium* species among symptomatic participants in the three study sites. **(C)** The proportions of mono and mixed infections of *Plasmodium* species among asymptomatic participants in the three study sites.

Unlike the symptomatic participants, the proportions for mono-infections for all the three *Plasmodium* species among the asymptomatic participants were comparable across the study sites ($P > 0.05$ for all comparisons, **Figure 6C**). *Pf/Pm* mixed infection in Kintampo was comparable to that in Sogakope ($\chi^2 = 3.19$, $P = 0.074$), but significantly lower than that in Cape Coast ($\chi^2 = 15.23$, $P < 0.0001$). On the contrary, *Pf/Po* mixed infections were found to be significantly higher in Kintampo compared to that in Cape Coast ($\chi^2 = 5.97$, $P < 0.015$) and Sogakope ($\chi^2 = 5.76$, $P = 0.016$). Taken together, the results show that the dynamics of *P. malariae* and *P. ovale* infections may vary across different malaria transmission sites and among different populations.

4.5.2 Age-dependent distribution of *Plasmodium* species

The prevalence of *P. falciparum*, *P. malariae* and *P. ovale* were further analysed based on age stratification (**Table 5**). Among the symptomatic participants, the prevalence of *P. falciparum* decreased with increasing age (**Figure 7**). On the contrary, a general increase in the prevalence of *P. malariae* and *P. ovale* infections with increasing age was observed (**Figure 7A**). There was no specific age-dependent pattern in the distribution of *P. falciparum*, *P. malariae* and *P. ovale* among the asymptomatic participants (**Figure 7B)**. The prevalence of mono and mixed infections were also analysed based on the age groups. Generally, the distribution of *P. falciparum* mono-infection among the symptomatic participants was found to be decreasing with increasing age (**Figure 7C**). Similarly, *Pf/Po* mixed infection was observed to be decreasing with increasing age but peaks after 40 years. Among the asymptomatic participants, no specific age-dependent pattern was observed in the prevalence of mono and mixed infections for all the three *Plasmodium* species **(Figure 7D**).

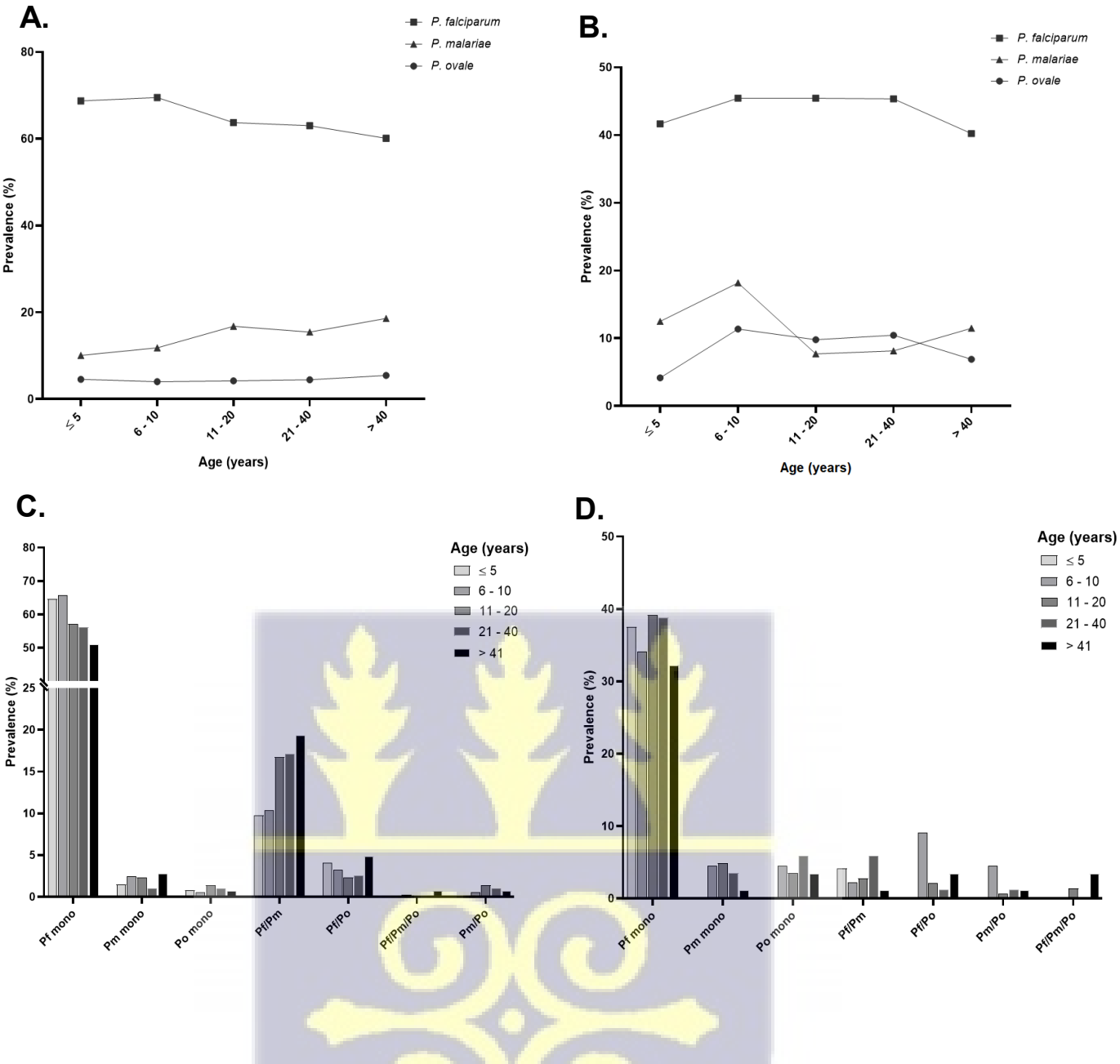

Figure 7: Age-dependent distribution of *Plasmodium* species.

Comparison of the prevalence of *P. falciparum* (Pf), *P. malariae* (Pm) and *P. ovale* (Po) based on age (years) stratification among symptomatic participants **(A)** and asymptomatic participants **(B)**. The prevalence of mono and mixed infections among study participants based on age-stratified analysis among symptomatic participants **(C)** and asymptomatic participants **(D)**. The prevalence was determined by expressing the total number of positive cases as a percentage of the total sample size for each age group.

4.5.3 Association between *Plasmodium* species infection and haematological parameters

The association of *P. falciparum*, *P. malariae* and *P. ovale* infections among symptomatic study participants on six haematological indices (haemoglobin, haematocrit, platelets, lymphocytes, monocytes and granulocytes) were assessed. Interestingly, the differences in the haemoglobin among participants with mono and mixed infections and those without *Plasmodium* species infection (participants who were negative for all the three *Plasmodium* species) did not reach statistical significance ($P > 0.05$ for all comparisons), even though haemoglobin levels were generally lower among participants with *Plasmodium* infection (**Figure 8A**). The haematocrit was found to be significantly lower among participants with *P. falciparum* mono-infection ($P = 0.002$) and *Pf/Pm* mixed infection ($P = 0.013$) compared to participants without *Plasmodium* species infection (**Figure 8B**). All other mono and mixed infections did not significantly affect the haematocrit when compared to the *Plasmodium*-negative participants ($P > 0.05$ for all comparisons; **Figure 8B**). Of note, both monocytes and granulocytes were not significantly affected by mono and mixed infections of *Plasmodium* species ($P > 0.05$ for all comparisons; **Figures 8C and 8D**). On the contrary, platelet counts were significantly lower among participants with *P. falciparum* mono-infection ($P < 0.0001$), *Pf/Pm* mixed infection ($P < 0.0001$), and *Pf/Pm/Po* mixed infection ($P = 0.002$) when compared to those without *Plasmodium* species infection (**Figure 8E**). Similarly, lymphocyte counts were found to be significantly lower among participants with *P. falciparum* mono-infection ($P = 0.003$), *Pf/Pm* mixed infection ($P = 0.002$), and *Pf/Pm/Po* mixed infection ($P = 0.002$) than that observed among those without *Plasmodium* species infection (**Figure 8F**).

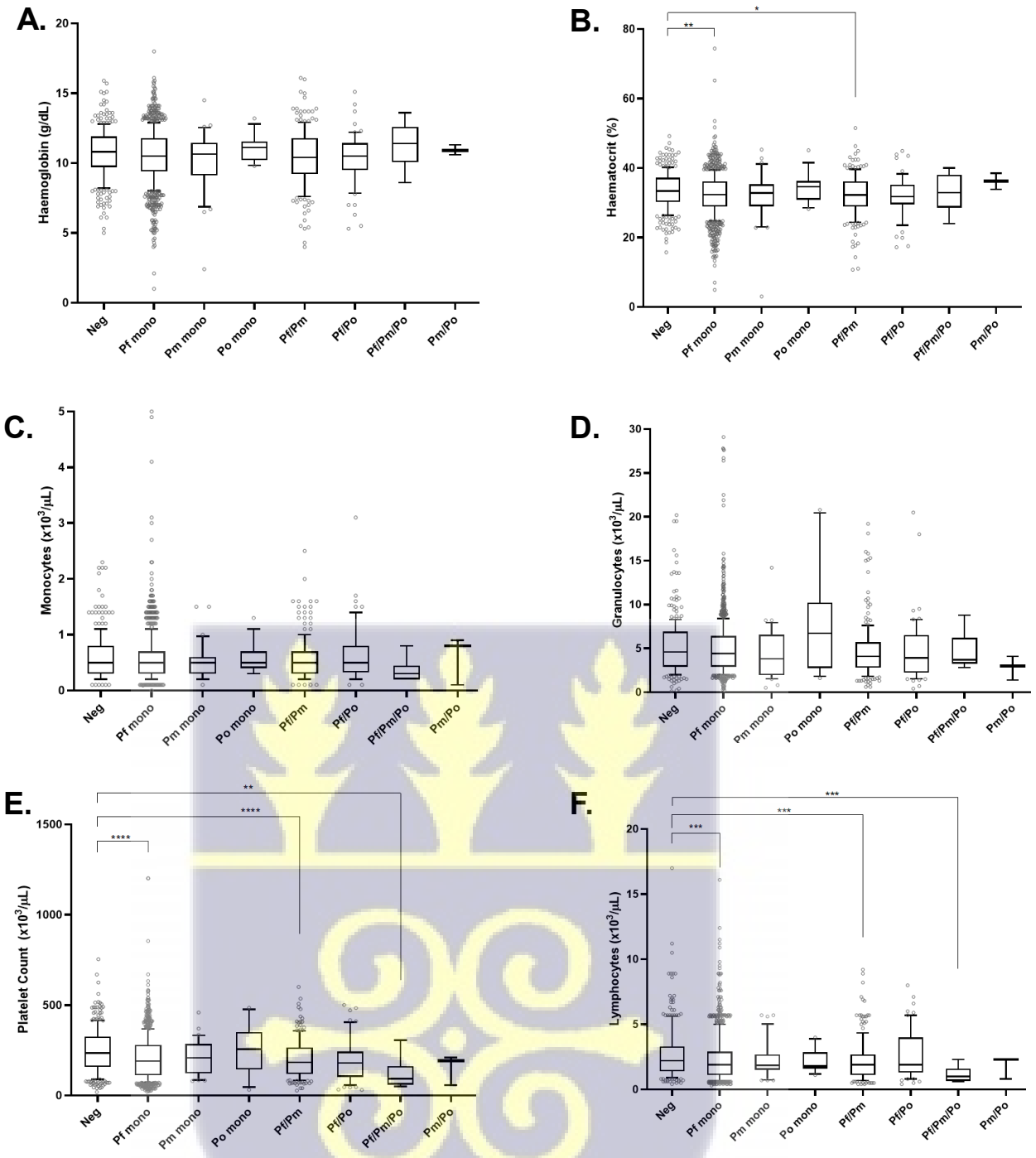

Figure 8: Association between *Plasmodium* species infections and haematological parameters.

Association of *Plasmodium* species infections with haemoglobin(**A**), haematocrit (**B**), monocytes (**C**), granulocytes (**D**), platelets (**E**) and lymphocytes (**F**). Clinical parameters among symptomatic study participants. Statistical significance has been donated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$. Data have been presented as box plot where the boxes represent the interquartile range, the line through the box is the median and the whiskers indicate the 10^{th} and the 90^{th} percentiles.

4.5.4 Correlation of parasite density with clinical parameters

Further to the association analysis, the three haematological indices that were significantly associated with mono and mixed *Plasmodium* infection (haematocrit, platelets and lymphocytes) were correlated with the copy numbers for each of the three *Plasmodium* species (**Figure 9**). An increase in *P. falciparum* copy number was found to be associated with corresponding reduction in haematocrit (Pearson's correlation coefficient, r = - 0.048, **Figure 9A**), platelets (r = - 0.093, **Figure 9B**) and lymphocytes (r = - 0.088, **Figure 9C**). Of note, *P. falciparum* copy number significantly correlated with platelets (P = 0.001) and lymphocytes (P = 0.002), but not haematocrit (P = 0.205). For non-falciparum species, both haematocrit (r = - 0.077) and lymphocyte count (r = - 0.015) negatively correlated with *P. malariae* copy number, while platelet count (r = 0.116) positively correlated with *P. malariae* copy number (**Figures 9D - 9F**). *P. ovale* copy number positively correlated with haematocrit (r = 0.061), platelets (r = 0.049) and lymphocytes (r = 0.180) counts (**Figures 9G – 9I**). However, none of the correlations between *P. malariae* and *P. ovale* copy numbers and the three haematological indices reached statistical significance (P > 0.05 for all comparisons).

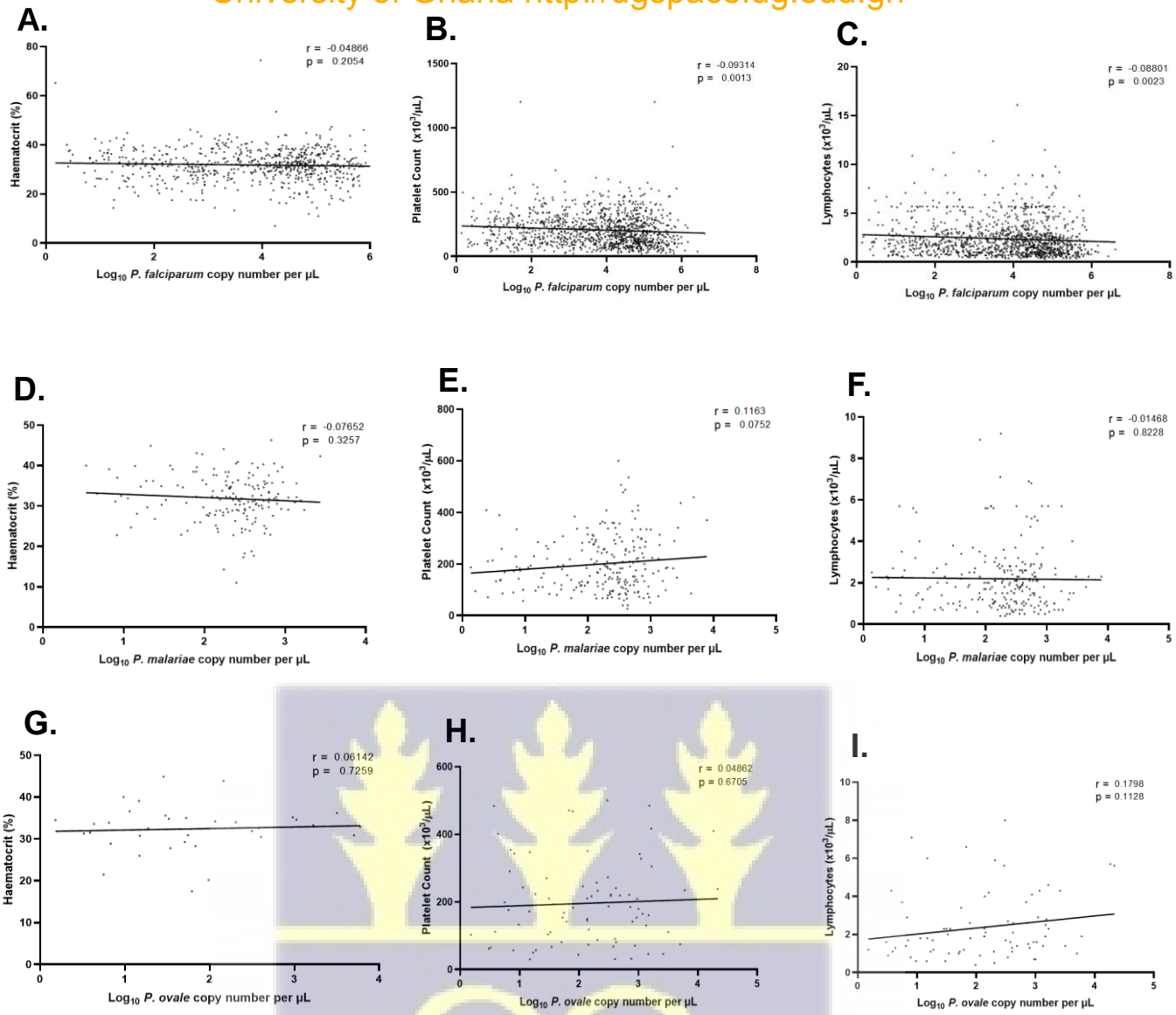

Figure 9: Correlation of parasite copy numbers with haematological parameters.

Correlation of *P. falciparum* copy number with haematocrit (**A**), platelets (**B**), lymphocytes (**C**). Correlation of *P. malariae* copy number with haematocrit (**D**), platelets (**E**), lymphocytes (**F**). Correlation of *P. ovale* copy number with haematocrit (**G**), platelets (**H**), lymphocytes (**I**). r and p represent Pearson's correlation coefficient and statistical significance (probability value), respectively.

4.5.5 Association between *Plasmodium* species infection and malarial anaemia

The associations between *Plasmodium* species infections and malarial anaemia were assessed (**Table 6**). Prior to this analysis, the associations between the demographic characteristics of study participants and severe anaemia were first investigated. The study participants were classified into three groups based on haemoglobin levels as previously described (World Health Organisation, 2011): without anaemia (i.e. Hb ≥ 11.0 g/dL), mild/moderate anaemia (11.0 > Hb > 7.0 g/dL), and severe anaemia (Hb ≤ 7.0 g/dL). Participants without anaemia (i.e. Hb ≥ 11.0 g/dL) were used as the reference group. The analyses showed that gender is not associated with mild/moderate anaemia (Adjusted Odds Ratio, AOR = 1.16, 95% confidence interval, CI = 0.93 - 1.44, *P* = 0.183) or severe anaemia (AOR = 0.87, 95% CI = 0.52 - 1.48, *P* = 0.617). For age-dependent analysis, study participants of ≤ 10 years were more likely to develop mild/moderate anaemia (*P* < 0.0001) and severe anaemia (*P* < 0.004) compared to older participants (**Table 6**). In the association between site and haemoglobin level, it was observed that participants in Kintampo had a lower risk of developing mild/moderate anaemia (AOR = 0.54, 95% CI = 0.38 - 0.76, *P* = 0.0004) compared to those from Cape Coast (**Table 6**). The differences in the haemoglobin level between participants from Cape Coast and Sogakope did not reach statistical significance (*P* ≥ 0.05; **Table 6**).

For association between *Plasmodium* infections and malarial anaemia, mono-infections of all of the three *Plasmodium* species were not significantly associated with the risk of developing mild/moderate (*P* > 0.05 for all comparisons; **Table 6**). Interestingly, participants with *Pf/Pm* (AOR = 1.75, 95% CI = 1.19 - 2.58, *P* = 0.005) and *Pf/Po* (AOR = 2.04, 95% CI = 1.07 - 3.89, *P* = 0.030) mixed infections had significantly higher risk of developing mild/moderate anaemia (**Table 6**). Study participants with *P. falciparum* mono infection (AOR = 5.52, 95% CI = 1.69 - 18.06, *P* = 0.005), *P. malariae* mono infection (AOR = 7.93, 95% CI = 1.19 - 52.81, *P* = 0.032) *Pf/Pm* mixed infection (AOR = 5.54, 95% CI = 1.42 - 21.66, *P* = 0.014) and *Pf/Po*

mixed infection (AOR = 9.06, 95% CI = 1.67 - 49.12, *P* = 0.011) had significant increased risk of developing severe anaemia (**Table 6**).

Table 6: Association between *Plasmodium* species infections and malarial anaemia.

Characteristic		Haemoglobin (Hb) level, g/dL					
		Mild or moderate anaemia (11.0 > Hb >7.0)			Severe anaemia (Hb ≤ 7.0)		
		AOR	**95% CI**	***P*-value**	**AOR**	**95% CI**	***P*-value**
Gender							
Male	Reference						
Female		1.16	(0.93-1.44)	0.183	0.87	0.52 - 1.48	0.617
Age (years)							
21 - 40	Reference						
0 – 5		3.76	2.67 - 5.31	<0.0001	10.48	2.45 - 44.78	0.002
6 - 10		2.93	2.03 - 4.23	<0.0001	8.656	1.97- 38.09	0.004
11 - 20		0.99	0.67 - 1.49	0.990	0.865	0.12 - 6.26	0.886
> 40		1.02	0.65 - 1.61	0.924	6.881	1.44 - 32.85	0.016
Study site							
Cape Coast	Reference						
Sogakope		0.92	0.67 - 1.25	0.584	2.63	1.00 - 6.92	0.05
Kintampo		0.54	0.38 - 0.76	0.0004	0.810	0.27 - 2.41	0.71
Infection							
Neg.	Reference						
Pf mono		1.29	0.98 - 1.70	0.072	5.52	1.69 - 18.06	0.005
Pm mono		1.73	0.76 - 3.94	0.193	7.93	1.19 - 52.81	0.032
Po mono		0.96	0.32 - 2.85	0.942	-	-	-
Pf/Pm		1.75	1.19 - 2.58	0.005	5.54	1.42 - 21.66	0.014
Pf/Po		2.04	1.07 - 3.89	0.030	9.06	1.67 - 49.12	0.011
Pf/Pm/Po		0.63	0.15 - 2.64	0.524	-	-	-
Pm/Po		2.36	0.20 - 28.34	0.498	-	-	-

AOR = adjusted odds ratio, CI = confidence interval, Neg. = Negative for *Plasmodium* species infection, Pf = *P. falciparum*, Pm = *P. malariae*, Po = *P. ovale*, mono = mono-infection. Associations between severe anaemia and Po mono, Pf/Pm/Po mixed infection, and Pm/Po mixed infection were not determined due to the small sample size.

4.6 Discussion and conclusion

This study determined the associations between *Plasmodium* species (*P. falciparum*, *P. malariae* and *P. ovale*) infections and haematological indices. To achieve this, the distribution of *P. falciparum*, *P. malariae* and *P. ovale* among symptomatic and asymptomatic participants were first determined. As expected, the prevalence of *P. falciparum* was significantly higher among symptomatic participants than asymptomatic participants. This observation parallels other studies in malaria-endemic regions, including Ghana (Owusu *et al*., 2017), Ethiopia (Mvumbi *et al*., 2016), and Tanzania (Sumari *et al*., 2017). Similarly, the difference in the frequency distribution of *P. malariae* was significantly higher in symptomatic participants than in asymptomatic participants. *P. ovale* infection, however, was comparable between symptomatic and asymptomatic participants. These results contrast the finding in a previous study in Indonesia which observed a higher prevalence of non-falciparum species among asymptomatic participants (Karyana *et al*., 2008). This observation may be due to differences in the transmission dynamics of the non-falciparum species among different populations.

Age-dependent acquisition of malaria immunity affects the dynamics of *Plasmodium* species infection (Aponte *et al*., 2007). In this study, *P. falciparum* infection was highest among participants aged 6 - 10 years and subsequently declined with increasing age as expected. This observation may be partly explained by the gradual loss of maternal protective immunity in the early years and subsequent acquisition of natural immunity in later years (Doolan *et al*., 2009; Simon *et al*., 2015). Both *P. malariae* and *P. ovale* prevalence were observed to be increasing with increasing age among symptomatic participants, which is consistent with previous studies in Burkina Faso (Gnémé *et al*., 2013) and Uganda (Betson *et al*., 2018). The observed relative increase in the prevalence of *P. malariae* and *P. ovale* with the corresponding decrease of *P. falciparum* prevalence may be due to the selection of non-falciparum species as a result of the *falciparum*-biased malaria interventions and control measures such as the use of artemisinin-

based combination therapies (ACTs) for both falciparum and non-falciparum uncomplicated malaria (Betson *et al*., 2014; Yman *et al*., 2019). In contrast to this observation, a previous longitudinal study in Senegal reported a near elimination of *P. malariae* and *P. ovale* as the prevalence of *P. falciparum* was decreasing (Roucher *et al*., 2014). These differences in the transmission dynamics of *Plasmodium* species underscore the importance of determining geographical site-specific transmission patterns for each *Plasmodium* species towards achieving effective control (Yman *et al*., 2019).

Plasmodium infections drive different degrees of clinical symptoms, of which anaemia is prime. Anaemia is defined by haemoglobin levels of <11 g/dL (White, 2018). Consistent with previous reports (Maina *et al*., 2010; Olliaro *et al*., 2011), participants in this study with *P. falciparum*, *P. malariae* and *P. ovale* infections generally had lower haemoglobin levels compared to *Plasmodium*-negative participants; however, the differences were not statistically significant. Interestingly, based on multivariate logistic regression analysis, it was observed that mono-infections of *P. falciparum*, *P. malariae* and *P. ovale* were not significantly associated with the risk of developing mild or moderate anaemia. However, participants harbouring mixed infections of *P. falciparum* with either *P. malariae* or *P. ovale* had ~2-fold higher risk of developing mild or moderate anaemia. This observation supports the study hypothesis that mixed infection of two or more *Plasmodium* species is associated with severe malarial anaemia. Furthermore, participants with *P. malariae* mono-infection and mixed infections of *P. falciparum* with either *P. malariae* or *P. ovale* had the greatest risk of developing severe anaemia. The results presented here are in line with a previous study that observed that individuals with *P. malariae* infection or mixed infection of *Plasmodium* species had increased risk of developing anaemia compared to mono-infections of *P. falciparum* and *P. vivax* (Douglas *et al*., 2013). These results suggest that interactions between falciparum and non-falciparum *Plasmodium* species may enhance malaria severity (Douglas *et al*., 2013;

Maitland *et al*., 1996; Maitland *et al*., 1997; Mayxay *et al*., 2004; Price *et al*., 2001). However, it is important to highlight that the pathogenesis of malarial anaemia is multifactorial and remains poorly understood (Awandare *et al*., 2009; Casals-Pascual & Roberts, 2006; White, 2018).

Several studies have associated malaria with alteration in WBC counts (McKenzie *et al.*, 2005; Tobón-Castaño *et al*., 2015; Van-Wolfswinkel *et al.*, 2017; Kotepui *et al.*, 2020c). In this study, WBC counts were not significantly affected by *P. falciparum*, *P. malariae* and *P. ovale* infections (data not shown). This finding is consistent with previous studies, which observed no differences in WBC counts between malaria and non-malaria infected groups (Bashawri *et al*., 2002; Chiwakata *et al*., 2000; Maina *et al*., 2010). In contrast, other studies have reported either an increase (Adedapo *et al*., 2007; Ladhani *et al*., 2002) or a decrease (Beale *et al*., 1972; Erhart *et al*., 2004; Lathia & Joshi, 2004) in WBCs following *Plasmodium* infection. Monocytes play an important protective role during malaria infection (Ortega-Pajares & Rogerson, 2018). In one study, monocyte counts were positively correlated with parasitaemia among patients with *P. falciparum* uncomplicated malaria (Abdalla, 1988). Contrary to this observation, this study observed a negative correlation between *P. falciparum* copy number and monocytes; however, the association was not statistically significant. Our finding aligns with another study in which low monocyte counts were associated with *P. falciparum* infection (Maina *et al*., 2010). This study also observed a positive correlation between granulocytes and *P. falciparum*, *P. malariae* and *P. ovale* copy numbers, which is consistent with previous study that showed *P. falciparum* infection results in increased granulocytes (Olliaro *et al*., 2011). Lymphocyte counts for participants with *P. falciparum* and *P. malariae* infections were lower compared to those without *Plasmodium* infection. This observation corroborates the findings from previous studies (Erhart *et al*., 2004; Richards *et al*., 1998) and this may be due to the

sequestration of circulating lymphocytes in the spleen following *Plasmodium* infection (Wickramasinghe & Abdalla, 2000).

Thrombocytopenia is commonly associated with *Plasmodium* infection and has been suggested as a possible indicator for the diagnosis of *P. falciparum* malaria (Purbiya *et al*., 2018; Tangvarasittichai *et al*., 2016). As expected, the platelet count in this study was significantly reduced in participants with *P. falciparum* mono and mixed infections. This observation is in line with the hypothesis that mixed infection of *Plasmodium* species is associated with altered haematological parameters. In addition, this finding is consistent with previous studies which reported a negative relationship between platelet count and *P. falciparum* parasite density (Erhart *et al*., 2004; Maina *et al*., 2010). Although the platelet counts for mono-infections of *P. malariae* and *P. ovale* were not significantly affected, lower platelet counts were generally observed compared to those without *Plasmodium* infection. These observations support the notion that the use of thrombocytopenia for differential diagnosis of *P. falciparum* malaria may be inaccurate as low platelet low count is also associated with *P. vivax* (Erhart *et al*., 2004; Koltas *et al*., 2007), *P. malariae* and *P. ovale* infections.

One limitation of the current study is our inability to control for other confounding factors such as the genetic background of participants and other underlying conditions such as viral, bacterial and worm infections, which could also alter the levels of the haematological indices under consideration. Regardless of this limitation, the study provides essential data on the alteration of haematological parameters following *P. falciparum, P. malariae* and *P. ovale* infections. In conclusion, even though mono-infections of *P. malariae* and *P. ovale* infections did not significantly alter haematological indices, interactions between falciparum and non-falciparum *Plasmodium* species appear to enhance malaria severity. These findings underscore the importance of the readily availability of reliable and cost-effective species-specific tools for malaria diagnosis at the POC.

CHAPTER FIVE

5.0 Paper 3: Development of in-house loop-mediated isothermal amplification (LAMP) assays for cost-effective detection of *P. falciparum*, *P. malariae* and *P. ovale*.

Specific Aim 3

To develop in-house RT-LAMP assays for cost-effective nucleic acid-based diagnosis of P. falciparum, P. malariae and P. ovale at the point-of-care (POC).

Research question 1

What is the diagnostic performance of in-house LAMP assay for the detection of P. falciparum, P. malariae and P. ovale?

5.1 Rationale for Specific Aim 3

P. malariae and *P. ovale* are usually detected as low-density infections below the limits of detection of microscopy and RDTs, which are the most readily and cost-effective diagnostic tools available at the POC (Njiru, 2012). As such, reliable diagnosis of these non-falciparum species at the POC would require highly sensitive and specific detection tools (Berzosa *et al*., 2018; Hofmann *et al*., 2015). LAMP is a field-adaptable NAAT that has been described for the routine species-specific diagnosis at the POC (Becherer *et al*., 2020). Nonetheless, the current high cost of LAMP assay reagents, particularly the *Bst* polymerase component, is beyond the capacity of most malaria-endemic settings (Lucchi *et al*., 2010). As such, the development of in-house LAMP assays using locally produced *Bst* polymerase will be essential for cost-effective and reliable species-specific diagnosis of malaria. To address specific aim 3, *Bst*-LF polymerase was expressed, purified and used to develop real-time LAMP assays to detect *P. falciparum*, *P. malariae* and *P. ovale*. The in-house real-time LAMP assays were validated using the clinical samples used in Specific Aim 1 to validate the cooperative primer-based qPCR assays. The diagnostic performance of the in-house real-time LAMP assays were compared to the cooperative primer-based qPCR assays.

5.2 Abstract

Loop-mediated isothermal amplification (LAMP) is a field-adaptable nucleic acid-based test that has been described as a reliable tool for species-specific diagnosis of malaria at the point-of-care (POC). However, the current cost of LAMP assays has hampered the application of this tool for routine malaria diagnosis. As such, the development of in-house LAMP assays would immensely contribute towards the availability of cost-effective diagnostic assays, especially in resource-limited settings. In this study, *Bacillus stearothermophilus* Large Fragment (*Bst*-LF) polymerase was expressed, purified and used to develop SYBR Green real-time LAMP (RT-LAMP) assays for the detection of *P. falciparum*, *P. malariae* and *P. ovale*. The estimated limits of detection for the RT-LAMP assays for *P. falciparum*, *P. malariae* and *P. ovale* were 1.0 genomic DNA copy/µL, <1.0 genomic DNA copy/µL, and ~1.0 genomic DNA copies/µL, respectively. The in-house LAMP assays were further evaluated on clinical isolates using 560 purified genomic DNA that have been previously analysed by qPCR assays in our previous study (**Chapter 3**). Compared to the qPCR assays, the diagnostic sensitivities for the RT-LAMP assays were 95.2%, 96.8% and 97.5% for *P. falciparum*, *P. malariae* and *P. ovale*, respectively. The specificities for the *P. falciparum*, *P. malariae* and *P. ovale* RT-LAMP assays were 90.0%, 86.2% and 87.3%, respectively. Altogether, the study illustrates the potential application of the RT-LAMP assays for species-specific cost-effective malaria diagnosis, which will be necessary for informing appropriate antimalarial treatment and disease management.

5.3 Introduction

The availability of affordable and reliable tools for the diagnosis of malaria at the point-of-care (POC) is a crucial step toward effective disease management (Njiru, 2012). However, the most readily available detection tools for the diagnosis of malaria lack adequate sensitivity and specificity for accurate species-specific detection of *Plasmodium* species infections. As such, loop-mediated isothermal amplification (LAMP), a field-adaptable NAAT, has been described as a potential tool for routine diagnosis of malaria (Becherer *et al*., 2020). Compared to PCR, LAMP is a relatively quick and simple technique with minimal operational requirements (Becherer *et al*., 2020; Thompson & Lei, 2020). This technique amplifies target nucleic acid under isothermal conditions using a *Bacillus stearothermophilus* (*Bst*) polymerase with a strand displacement activity (Becherer *et al*., 2020). *Bst* polymerase is more robust to inhibition than *Taq* polymerase, which makes LAMP field-friendly with minimal nucleic acid purification requirement (Modak *et al*., 2016). In addition, the resulting LAMP product can be visualised with the naked eye based on a change in reaction turbidity resulting from magnesium pyrophosphate precipitate or the use of detection dye such as hydroxy-naphthol blue (HNB) (Lau *et al*., 2016; Port *et al*., 2014).

Several LAMP-based assays have been reported for the detection of *Plasmodium* species with limit of detection of <10 parasites/µL of template DNA (Hopkins *et al*., 2013; Lau *et al*., 2016; Mohon *et al*., 2014; Polley *et al*., 2010). Despite these advantages, the current high cost of the LAMP reagents, particularly the *Bst* polymerase, has been a major setback towards the deployment of LAMP for routine diagnosis of malaria (Becherer *et al*., 2020). In pursuance of affordable and species-specific diagnosis, local production of *Bst* polymerase for the development of LAMP assays would be a major step towards the availability of reliable nucleic acid-based amplification assays for species-specific diagnosis of malaria at the POC. In this study, *Bst* Large Fragment (*Bst*-LF) polymerase was expressed, purified, and used to develop

SYBR Green-based real-time LAMP (RT-LAMP) assays for the detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical assays.

5.4 Materials and Methods

5.4.1 Expression of *Bst*-LF polymerase

The pET21a(+)-Bst LF-6xHis (Addgene plasmid # 159148; http://n2t.net/addgene:159148; RRID: Addgene_159148) expression plasmid was received from Andrea Pauli (Research Institute of Molecular Pathology, Vienna). The plasmid was transformed into BL21 (DE3) competent *Escherichia coli* cells (Biolabs, New England, USA) and incubated overnight at 37°C on an agar selection plate containing 50 µg/mL ampicillin. A single colony was selected and inoculated into 20 mL LB broth containing 50 µg/mL ampicillin and then incubated overnight at 37 °C with shaking at 225 rpm. The overnight culture was used as a starter culture to inoculate fresh 200 mL LB broth containing 50 µg/mL ampicillin. The resulting culture was incubated at 37°C to an optical density (OD_{600}) of ~0.6 and then induced with 1.0 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was further incubated for 6 hours at 37°C in a shaking incubator at 225 rpm. The resulting cells were harvested at 5,000 rpm for 20 minutes and stored at -20 °C until ready for purification.

5.4.2 Purification of *Bst*-LF polymerase

The harvested cell pellets were resuspended in a volume of 10 mL lysis buffer (50 mM of Tris-hydrochloric acid (HCl), pH 7.9; 50 mM of dextrose, 1 mM of ethylenediaminetetraacetic acid (EDTA)) and then subjected to two runs of freeze-thaw (freezing at -80°C and thawing at 37°C). The resulting lysate was further subjected to 30 cycles of sonication (10 seconds on and 5 seconds off) using the QSONICA sonicator (Qsonica, Connecticut, USA) at an amplitude of 40%. The lysate was then centrifuged at 13,000 rpm for 30 minutes in a refrigerated centrifuge. A volume of 1 mL of the supernatant was applied to 0.3 mL ProBond™ Nickel-Chelating Resin (Thermo Fisher Scientific, UK) that has been pre-equilibrated with the wash buffer (20 mM

Tris-HCl pH 7.5, 300 mM sodium chloride (NaCl) and 20 mM imidazole). The mixture was incubated at room temperature with gentle rotation for 30 minutes and then centrifuged at 10,000 rpm for 5 minutes. The resulting ProBond™ Nickel-Chelating Resin was washed 3 times with excess wash buffer, and the *Bst*-LF polymerase was eluted with 200 mL elution buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl and 250 mM imidazole). The eluted *Bst*-LF polymerase was desalted and concentrated with the 50 kDa Amicon Ultra Centrifugal Filter cut-off column (Sigma Aldrich, UK). The resulting *Bst*-LF polymerase fraction was suspended in 1 mL of storage buffer (10 mM Tris-HCl at pH = 7.4, 50 mM potassium chloride (KCl), 1 mM dithiothreitol of (DTT), 0.1 mM EDTA, 0.1% Triton X-100 and 50% glycerol) and stored at -20^{o}C until ready for molecular assays.

5.4.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis for *Bst*-LF polymerase

The cell lysate, purification fractions and the purified *Bst*-LF polymerase were analysed on a 4–12% mini-Protean TGX Precast Gel (BioRad, California, USA). The samples were prepared in 1X Laemmli buffer (20% β-ME, 40% glycerol, 8% SDS, 0.008% bromophenol blue, 0.25 M Tris HCl, pH 6.8) and incubated at 94 ℃ for 10 minutes. A volume of 30 µL of each sample was separated on the precast gel for 90 minutes at a voltage of 100 V. The resulting gel was stained with SimplyBlue™ Safe Stain (Thermo Scientific), and the image was processed using the Amersham 600 imager (General Electrics Healthcare Life Sciences, Chicago, USA). For western blot analysis, the separated proteins were transferred onto a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (BioRad, California, USA). The resulting membrane was washed twice in Tris-buffered saline (TBS) and then blocked with 3% Bovine Serum Albumin for 60 minutes at room temperature. The membrane was further washed in

TBS-Tween/Triton buffer and incubated with 1:2000 diluted Anti-His HRP Conjugate solution (Qiagen, Manchester, UK) for 60 minutes at room temperature. Following this, the membrane was further washed twice in excess TBS-Tween/Triton buffer and then enhanced using chemiluminescence reagent A (2.5 mM luminol, 400 µM p-coumaric acid, 100 mM Tris-HCl pH 8.5) and reagent B (0.018% H_2O_2, 100 mM Tris-HCl pH 8.5) (ThermoFisher Scientific, UK). The image of the resulting nitrocellulose membrane was processed using the Amersham 600 imager.

5.4.4 Development of RT-LAMP assays using purified *Bst*-LF polymerase

To validate the activity of the purified *Bst* polymerase, SYBR Green-based RT-LAMP assays were developed for the detection of *P. falciparum*, *P. malariae* and *P. ovale*. The assays were performed on the QuantStudio5 system (Applied Biosystems, UK) using previously reported primer sets (**Table 7**). All reactions were performed in a total volume of 20 µL consisting of 1X in-house amplification buffer (20 mM Tris-base at pH = 8.8, 50 mM potassium chloride (KCl), 10 mM ammonium sulphate [$(NH_4)_2SO_4$], 2.0 mM magnesium sulphate ($MgSO_4$), and 0.1% Tween 20), 6.0 mM magnesium chloride ($MgCl_2$), 400 mM betaine, 1.5 mM deoxyribonucleotide triphosphates (dNTPs), 1.0 µM SYTO-9 dye, 1X primer mix solution containing 1.5 µM of forward inner primer (FIP), 1.5 µM of backward inner primer (BIP), 0.4 µM of forward loop primer (FLP), 0.4 µM of backward loop primer BLP, 0.2 µM of forward outer primer (F3), and 0.2 µM of backward outer primer (B3), 0.5 µL of the purified *Bst* polymerase and 3 µl of template DNA. The reaction was performed at 65°C for 35 minutes with fluorescence readings every 60 seconds on the SYBR Green channel. The specificity of the resulting amplicons was determined by analysing the melting curves.

Table 7: List of primers used for the in-house RT-LAMP assays.

Target	Primer code	Sequence (5′→3′)	Reference
P. malariae	Pm_F3	CAAGGCCAAATTTTGGTT	(Han *et al*., 2007)
	Pm_B3	CGGTTATTCTTAACGTACA	
	Pm_FIP	TATTGGAGCTGGAATTACCGCGATGATGGGAATTTAAAACCT	
	Pm_BIP	AATTGTTGCAGTTAAAACGCCTATGTTATAAATATACAAAGCATT	
	Pm_LPF	GCCCTCCAATTGCCTTCTG	
	Pm_LPB	TCGTAGTTGAATTTCAAGGAATCA	
P. ovale	Po _F3	GGAATGATGGGAATTTAAAACC	(Han *et al*., 2007)
	Po _B3	GAATGCAAAGAACAGATACGT	
	Po_FIP	TATTGGAGCTGGAATTACCGCGTTCCCAAAATTCAATTGGAGG	
	Po_BIP	GTTGCAGTTAAAACGCTCGTAGTGTATTGTCTAAGCATCTTATAGCA	
	Po_LPF	TGCTGGCACCAGACTTGC	
	Po _LPB	TGAATTTCAAAGAATCAA	
P. falciparum	Pf_F3	CTCCATGTCGTCTCATCGC	(Polley *et al*., 2010)
	Pf_B3	AACATTTTTTAGTCCCATGCTAA	
	Pf_FIP	ACCCAGTATATTGATATTGCGTGACAGCCTTGCAATAAATAATATCTAGC	
	Pf_BIP	AACTCCAGGCGTTAACCTGTAATGATCTTTACGTTAAGGGC	
	Pf_LPF	CGGTGTGTACAAGGCAACAA	
	Pf_LPB	GTTGAGATGGAAACAGCCGG	

Pm = *P. malariae*, Po = *P. ovale*, Pf = *P. falciparum*, FIB = forward inner primer, BIP = backward inner primer, FLP = forward loop primer, BLP = backward loop primer, F3 = forward outer primer (F3) and B3 = backward outer primer.

5.4.5 Analytical specificity and limit of detection of in-house RT-LAMP assays

The experimental specificity and the limits of detection (LODs) of the in-house RT-LAMP assays were determined. The specificity of the assays was determined using genomic DNA of *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. The LODs of the assays were estimated from standard curves obtained from serial dilutions of the *P. falciparum*, *P. malariae* and *P. ovale* genomic DNA. Each parasite genomic DNA was serially diluted to concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 and 10^{-1} copies/µL in TE buffer.

5.4.6 Detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical samples

The in-house RT-LAMP assays were further validated for the detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical isolates using 560 purified DNA samples that have been previously analysed (**Chapter 3**). The RT-LAMP assays were performed as previously described earlier in this study (section 5.4.4). The specificity of the resulting amplicons was determined by analysing the melting curves, and the resulting threshold cycle (C_t) values were used to estimate parasite copy number/µL.

5.4.7 Diagnostic Sensitivity and Specificity

The diagnostic sensitivity and specificity of the RT-LAMP assays were determined using the cooperative primer-based qPCR assays reference method. The diagnostic sensitivity was defined as the percentage of true positives out of all the participants who were positive based on the reference method (Becherer *et al*., 2020; Shreffler & Huecker, 2020). This was achieved using the equation, Sensitivity = (True positives)/ [(True positives + False negatives)] (Parikh *et al*., 2008; Shreffler & Huecker, 2020; Trevethan, 2017). The diagnostic specificity was also defined as the percentage of true negatives out of all the participants who were negative based

on the reference method (Becherer *et al*., 2020; Shreffler & Huecker, 2020). The diagnostic specificity was determined using the equation, Specificity = (True negatives)/ [(True negatives + False positives)] (Parikh *et al*., 2008; Shreffler & Huecker, 2020; Trevethan, 2017).

5.4.8 Statistical analyses

Data analysis was performed using GraphPad Prism 8.0.2 and Microsoft Excel software. Probit analysis was used to estimate the LOD of the assays at 95% confidence interval. Statistical significance for the proportions of positive samples between the RT-LAMP and qPCR assays were determined using the Chi-Square test. Comparison of the time-to-positivity for the assays was performed using Student's t-test. Correlation analysis of C_t-values for the assays was conducted using Pearson's product-moment correlation. Statistical significance for all analyses was considered at *P*-value less than 0.05.

5.5 Results and Discussion

5.5.1 Expression and purification of *Bst*-LF polymerase

In this study, *Bst*-LF polymerase was expressed, purified and used for the development of in-house RT-LAMP assays. The *Bst*-LF polymerase was expressed using a pET21a vector containing a T7 promoter with transcriptional and translational regulatory systems that are tightly regulated, which allow for high expression of the target gene (Oliveira *et al*., 2001; Ramos *et al*., 2004; Studier & Moffatt, 1986; Studier *et al*., 1990). As shown in **Figure 10A**, separation of the cell lysate following IPTG induction indicates successful expression and purification of the *Bst*-LF polymerase with the expected molecular weight of ~66.6 kDa. The purified *Bst*-LF polymerase was further confirmed using western blot analysis (**Figure 10B**).

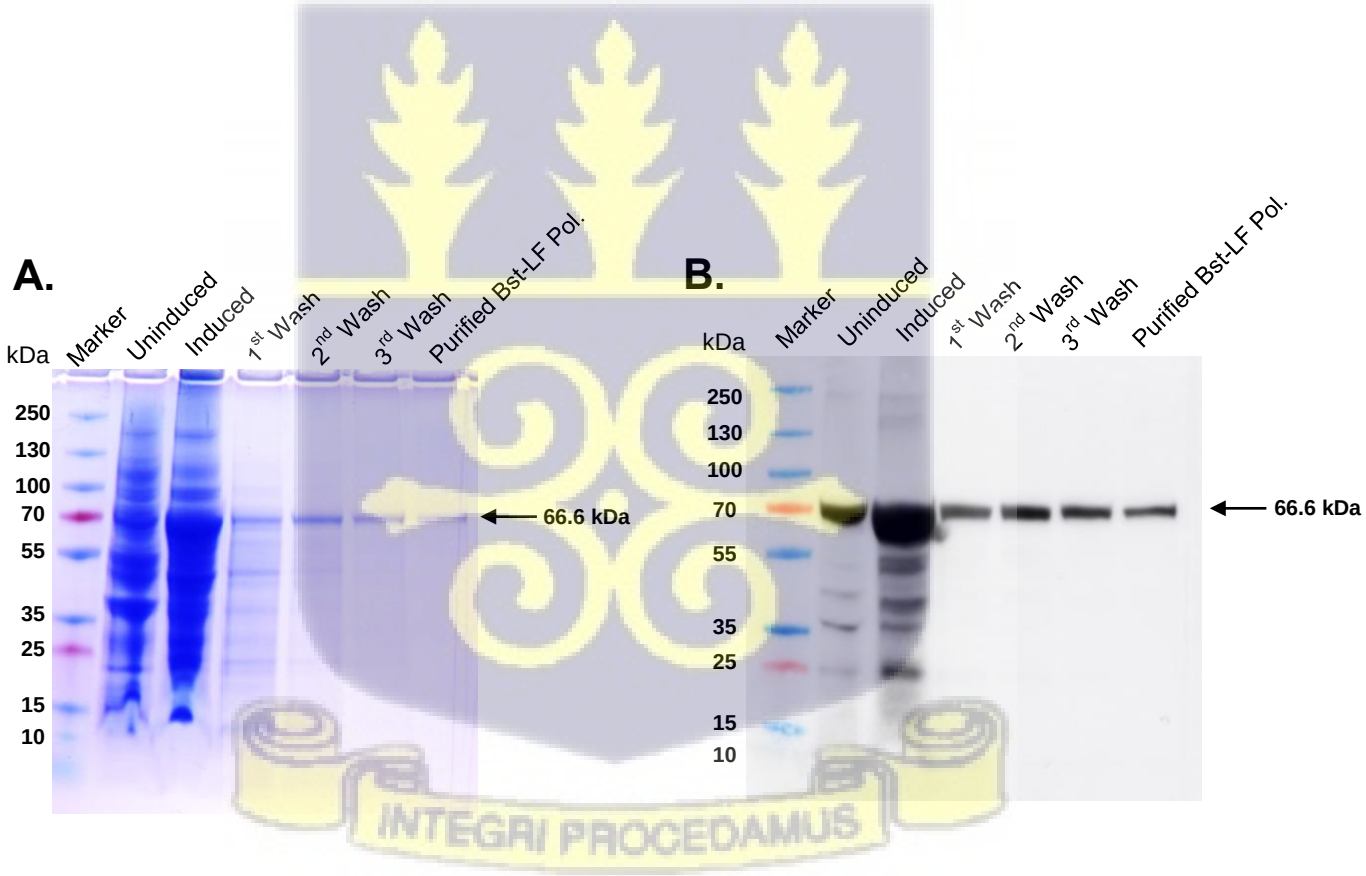

Figure 10: SDS-PAGE gel and western blot analysis of *Bst*-LF polymerase.

(A) SDS-PAGE gel showing separation of cell lysates before and after IPTG induction, purification fractions and the purified *Bst*-LF polymerase. (**B)** Western blot image showing successful expression and purification of *Bst*-LF polymerase. The expected molecular weight for *Bst*-LF polymerase was 66.6 kDa.

5.5.2 Development of SYBR Green-based RT-LAMP assays

The purified *Bst* polymerase was used to develop SYBR Green-based RT-LAMP assays for the detection of *P. falciparum*, *P. malariae* and *P. ovale*. Using serially diluted genomic DNA ranging from 10^6 to 10^{-1} parasite copies/µL, the LODs of the in-house RT-LAMP assays were determined (**Figures 11A – 11C**). The estimated LODs for the RT-LAMP assays for *P. falciparum*, *P. malariae* and *P. ovale* were ~1.0 genomic DNA copy/µL, < 1.0 genomic DNA copy/µL and ~1.0 genomic DNA copies/µL, respectively (**Figures 12A - 12C**). Of note, the LODs for the *P. malariae* RT-LAMP assay was comparable to that of the *P. malariae* cooperative primer-based qPCR assay. On the other hand, the LOD of the RT-LAMP assay for *P. ovale* was approximately 10-fold higher than that of the *P. ovale* cooperative primer-based qPCR assay. The observed LODs of the current RT-LAMP assays are comparable to the LODs that have been reported for commercially available LAMP kits for the detection of *Plasmodium* species (De-Koninck *et al.*, 2017; Han *et al.*, 2007; Lucchi *et al.*, 2016; Polley *et al.*, 2010). This observation suggests that the RT-LAMP assays would be suitable for the species-specific detection of low-density *P. falciparum*, *P. malariae* and *P. ovale* infections.

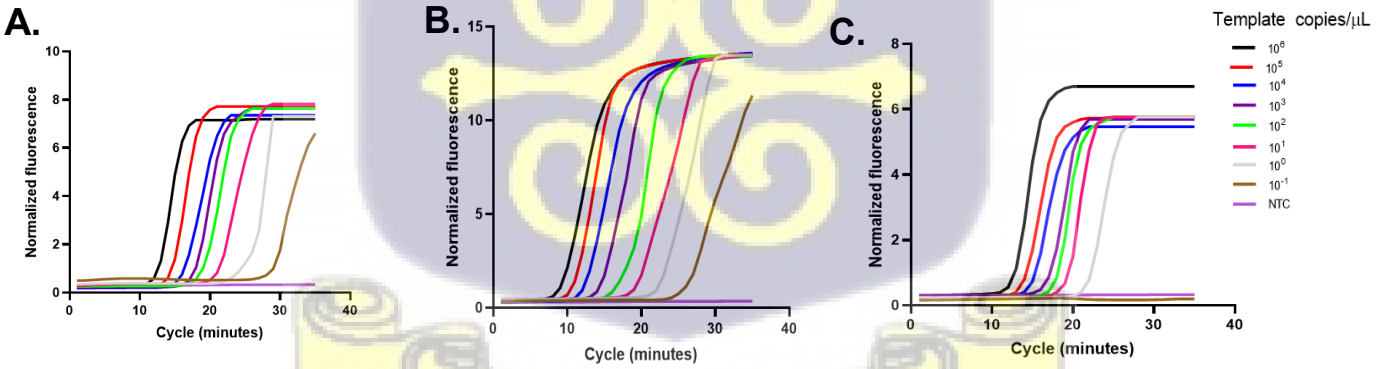

Figure 11: The sensitivity of the RT-LAMP assays.

Amplification plots for in-house RT-LAMP SYBR Green-based assay for *P. falciparum* (**A**), *P. malariae* (**B**) and *P. ovale* (**C**) using serially diluted parasite genomic DNA ranging from 10^6 to 10^{-1} genomic DNA copies/µL

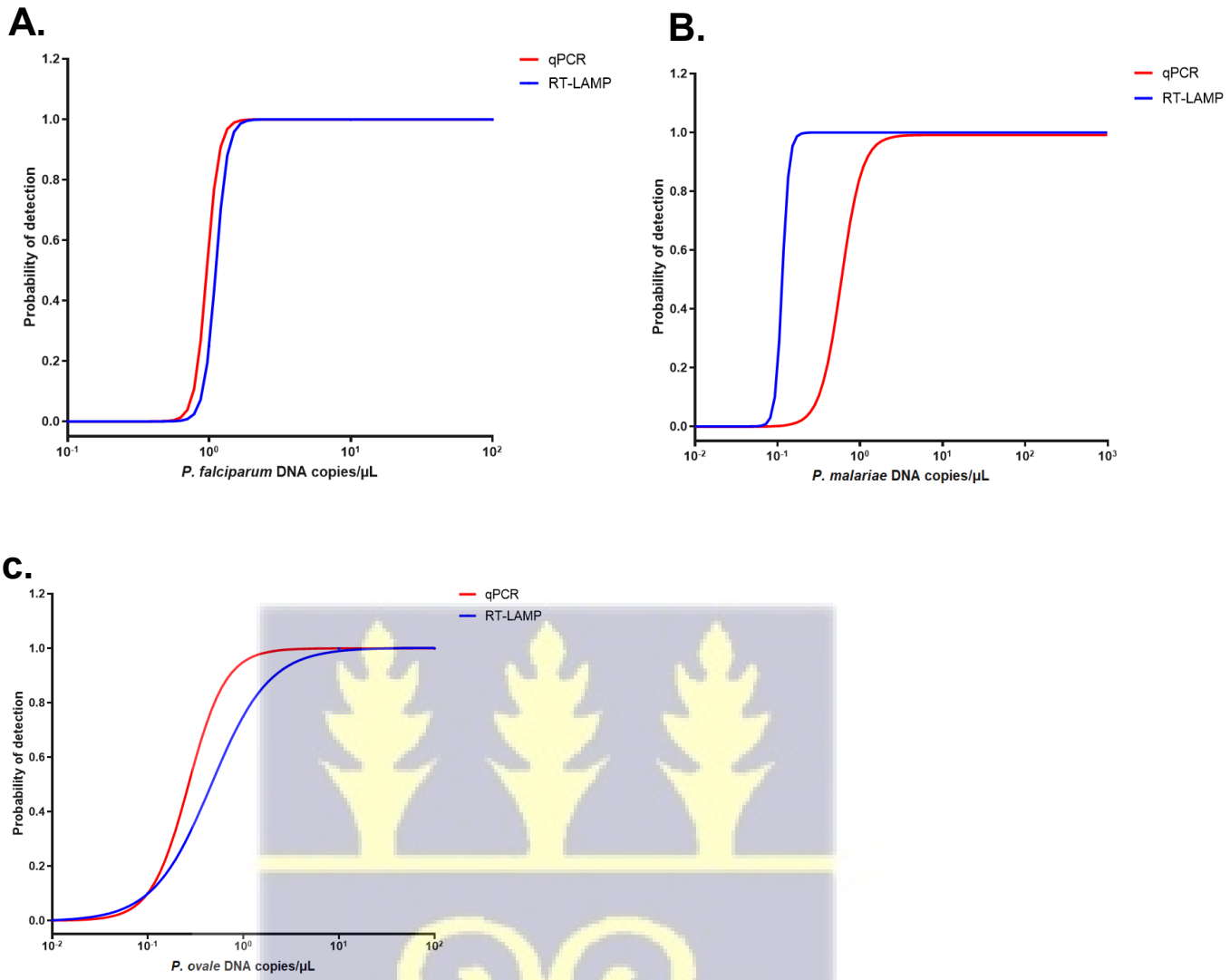

Figure 12: Comparison of the RT-LAMP and the qPCR assays using Probit analysis

The probability plots for the estimation of the limit of detection for *P. falciparum* **(A)**, *P. malariae* **(B)** and *P. ovale* **(C)** in-house RT-LAMP assays (blue) compared to the qPCR assays (red). Limits of detection were estimated from the 10-fold serially diluted parasite genomic DNA as reaction template.

5.5.3 Detection of *Plasmodium* species in clinical isolates using the in-house RT-LAMP assays

The RT-LAMP assays were validated for the detection of *P. falciparum*, *P. malariae* and *P. ovale* using 560 clinical samples that have been previously analysed using qPCR assays (**Chapter 3**). The prevalence rates of *P. falciparum*, *P. malariae* and *P. ovale* by the RT-LAMP were 94.3% (528/560), 23.2 % (130/560) and 14.8% (83/560), respectively (**Figure 13A**). The differences in the distribution of *P. falciparum* (Chi-square, χ^2 = 0.447, *P* = 0.504) and *P. malariae* (χ^2 = 3.632, *P* = 0.056) as determined by the RT-LAMP assays were comparable to those obtained using the qPCR assays. On the other hand, the distribution of *P. ovale* (χ^2 = 26.410, *P* < 0.0001) was significantly higher using the RT-LAMP than that obtained by the qPCR assay. The diagnostic sensitivity and specificity of the RT-LAMP assays were determined using the qPCR assays as the reference method. The sensitivities of the *P. falciparum*, *P. malariae* and *P. ovale* RT-LAMP assays were 95.2%, 96.8% and 97.5%, respectively, while the specificities were 90.0%, 86.2% and 87.3%, respectively (**Table 8**). The results presented here indicate that the diagnostic performance of the in-house RT-LAMP assays are within the range or comparable to those that have been previously reported for LAMP assays for the detection of *Plasmodium* species (Morris & Aydin-Schmidt, 2021; Picot *et al*., 2020; Roth *et al*., 2016; Selvarajah *et al*., 2020).

The C_t-values obtained from the RT-LAMP assays were compared to the C_t-values for the qPCR assays. The C_t-values of the RT-LAMP assays positively correlated with those of the qPCR assays (r = 0.694, *P* < 0.0001) (**Figure 13B)**. The mean C_t-values (time-to-positive) for the RT-LAMP and the qPCR assays were 15.21 ± 0.35 minutes and 21.37 ± 0.61 minutes, respectively (**Figure 13C**). As expected, the average time-to-positive for the RT-LAMP assays was significantly lower than that of the qPCR assays (Student's t-test = 21.69, *P* < 0.0001) (**Figure 13D**).

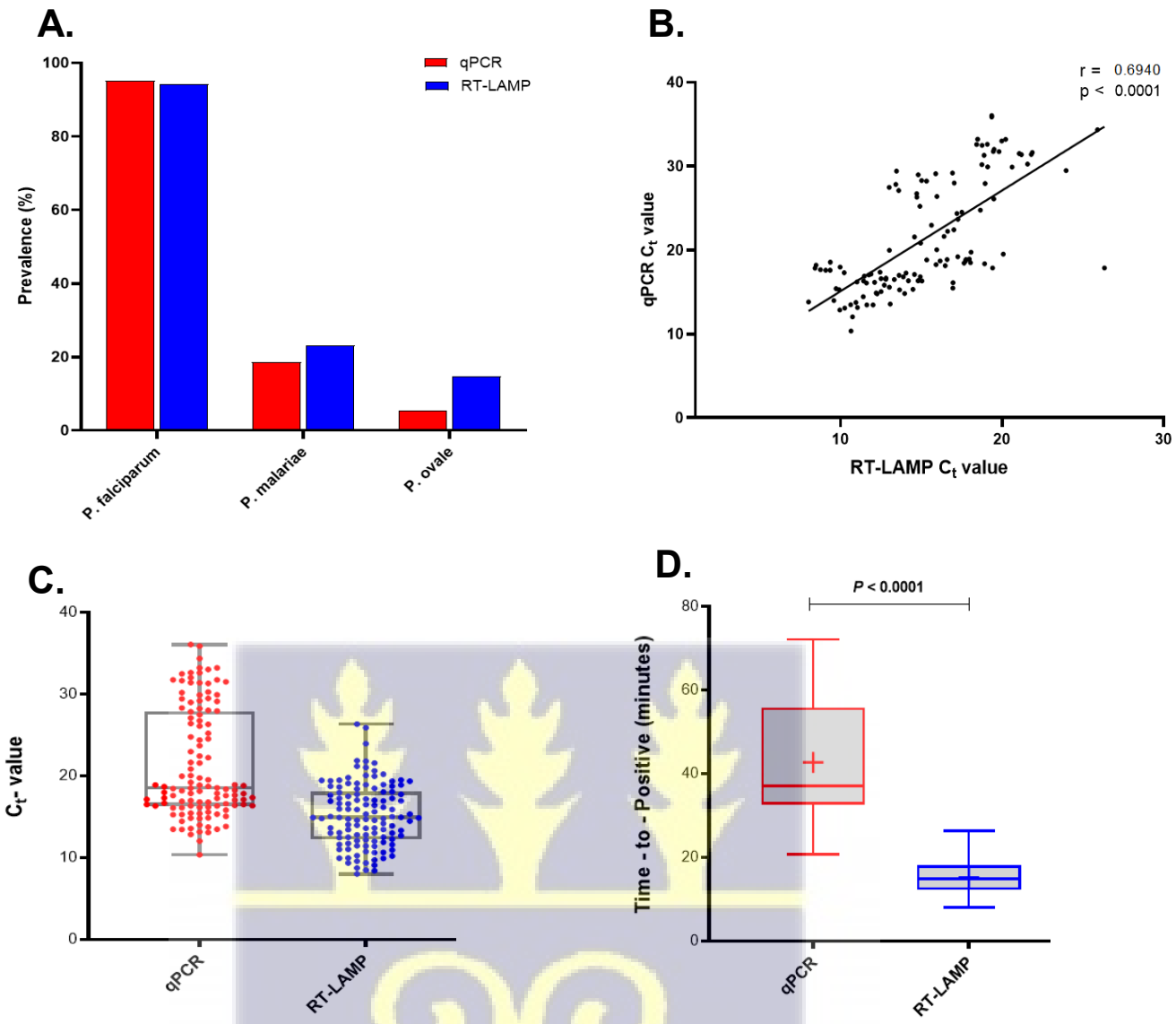

Figure 13: Comparison of the prevalence of *Plasmodium* species and the C_t-values for the RT-LAMP and the qPCR assays.

(A) Prevalence of *P. falciparum*, *P. malariae* and *P. ovale*. **(B)** Correlation of the C_t-values for the RT-LAMP and the qPCR assays. **(C)** C_t-values for the RT-LAMP and the qPCR assays. The dots represent the C_t-values for the individual positive samples. **(D)** The time-to-positive for the RT-LAMP and the qPCR assays.

Table 8: Summary of the analytical performance of the RT-LAMP assays compared to the qPCR assays.

***Plasmodium* species**	**Assay**	**Sensitivity (%)**	**Specificity (%)**
	qPCR	Reference	Reference
P. falciparum	RT-LAMP	95.2	90.0
P. malariae	RT-LAMP	96.8	86.2
P. ovale	RT-LAMP	97.5	87.3

qPCR = real-time quantitative polymerase chain reaction, RT-LAPM = real-time loop-mediated isothermal amplification

5.6 Conclusion

Taken together, the data presented here indicate that the RT-LAMP assays have high sensitivity and specificity for the detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical isolates. Further studies for the translation of the current LAMP assays into portable colorimetric diagnostic kits that allows easy visualisation of the resulting LAMP product with the naked eye would be instrumental in establishing affordable species-specific diagnosis of malaria at the POC. In addition, pilot implementation testing of the LAMP assay would be important to properly estimate the cost for POC applications, and also identify real-world challenges that may be associated with the LAMP assay in clinical or field settings.

CHAPTER SIX

6.0 Paper 4: Development of DNA-based electrochemical biosensors for ultrasensitive detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical samples.

Specific Aim 4

To develop label-free DNA-based electrochemical biosensors for the detection of P. falciparum, P. malariae and P. ovale in clinical isolates.

Research question 2

What is the diagnostic sensitivity and specificity of electrochemical biosensors for the direct of detection of unamplified P. falciparum, P. malariae and P. ovale genomic DNA in clinical isolates?

6.1 Rationale for Specific Aim 4

Biosensors are field-adaptable diagnostic devices with quick turnaround time, low operational cost, and require less technical expertise (LaBarre *et al*., 2011; Wang, 2008). Currently, the biosensors that have been reported for malaria diagnosis are mainly based on antigen-antibody interaction with *P. falciparum* histidine-rich protein II (PfHRP2) and *Plasmodium* lactate dehydrogenase (pLDH) being the most common antigenic biomarkers (Krampa *et al*., 2020). Of note, these biomarkers lack adequate sensitivity and specificity for the diagnosis of both falciparum and non-falciparum malaria (Agaba *et al*., 2019; Simpalipan *et al*., 2018; Verma *et al*., 2018). Alternatively, nucleic acid biomarkers offer highly sensitive and specific diagnosis (Cordray & Richards-Kortum, 2012). In addition, the use of label-free electrochemical detection methods allows for the development of cost-effective and scalable diagnostic devices (Kanyong *et al*., 2020a). To address specific aim 4, micro-gold electrodes were designed and commercially acquired. The electrodes were used to develop DNA-based biosensors based on an impedance transducing mechanism to detect *P. falciparum*, *P. malariae* and *P. ovale* parasites. The biosensors were validated to for the direct detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical isolates using purified genomic DNA and whole blood lysates selected from the larger sample pool previously analysed by qPCR assays. The results for specific aim 4 have been published in *Electrochimica Acta* journal;

https://doi.org/10.1016/j.electacta.2022.140988

6.2 Abstract

The absence of reliable species-specific diagnostic tools for malaria at point-of-care (POC) remains a major setback towards effective disease management. This is partly due to the limited sensitivity and specificity of the current malaria POC diagnostics especially in cases of low-density parasitaemia and mixed species infections. In this study, we describe the first label-free DNA-based biosensors based on electrochemical impedance spectroscopy (EIS) for species-specific detection of *P. falciparum*, *P. malariae* and *P. ovale*. The limits of detection (LODs) for the three species-specific biosensors were down in attomolar concentrations ranging from 18.7 aM to 43.6 aM, which is below the detection limits of previously reported malaria biosensors. More importantly, the diagnostic performance of the three biosensors were compared to quantitative real-time polymerase chain reaction (qPCR) assays using purified genomic DNA and the paired whole blood lysates from clinical samples. Remarkably, all the qPCR-positive purified genomic DNA samples were correctly identified by the biosensors indicating 100% sensitivity for each of the three malaria species. The specificities of the three biosensors ranged from 66.7% to 100.0% with a Therapeutic Turnaround Time (TTAT) within 30 minutes, which is comparable to the TTAT of current POC diagnostic tools for malaria. This work represents a significant step towards the development of accurate and rapid species-specific nucleic acid-based toolkits for the diagnosis of malaria at the POC.

6.3 Introduction

The absence of reliable tools for species-specific diagnosis of malaria at the POC is a major challenge for appropriate antimalarial treatment and disease management. This challenge calls for the availability of cost-effective, easy-to-use and rapid diagnostic tools at the POC, especially in resource-limited settings. In recent years, the application of biosensors as diagnostic devices at the POC has received considerable attention. Biosensors have high sensitivity and specificity, quick turnaround time, low operational cost, and require less technical expertise (LaBarre *et al*., 2011; Wang, 2008).

Several biosensors have been reported for malaria diagnosis using various parasite biomarkers, including antigens, antibodies, nucleic acids and infected red blood cells (Krampa *et al*., 2020). Among these biomarkers, parasite antigens are the most widely used, with *P. falciparum* histidine-rich protein 2 (PfHRP2) and genus *Plasmodium* lactate dehydrogenase (pLDH) being the most common analytes (Krampa *et al*., 2017). However, the reliability of these biomarkers has been limited by their global genetic diversity for both PfHRP2 and pLDH, and also the persistence detection of PfHRP2 several days following antimalarial treatment (Agaba *et al*., 2019; Simpalipan *et al*., 2018; Verma *et al*., 2018). In addition, these biomarkers lack specificity for species-specific detection of non-falciparum species.

Alternatively, nucleic acid-based biomarkers provide superior analytical performance (high sensitivity and specificity) for the detection of the target of interest (Cordray & Richards-Kortum, 2012). In addition, nucleic acid-based biorecognition receptors are relatively easy to identify, cost-effective and have high stability. Despite these advantages, only a few biosensors have been reported for nucleic acid-based detection of *Plasmodium* species (Krampa *et al*., 2020). The first DNA-based malaria biosensor was developed for the detection of *P. falciparum* based on quartz crystal microbalance (QCM) using gold electrodes (Potipitak *et al*., 2011). To reduce the operational cost, the same group subsequently described another QCM DNA-based

biosensors for the detection of *P. falciparum* and *P. vivax* using silver electrodes (Wangmaung *et al*., 2014). However, these biosensors require initial PCR amplification of the target DNA, which limits their application for routine diagnosis at the POC. As such, Ngo *et al*. described a highly sensitive DNA-based biosensor based on surface-enhanced Raman scattering (SERS) for rapid detection of *P. falciparum* without the need for pre-amplification of target DNA (Ngo *et al*., 2016). Despite the high sensitivity, SERS-based biosensors require cumbersome multiple steps, large sample volume, and reagents which render them less suitable for routine POC application (Liu *et al*., 2019).

In this study, we developed the first label-free DNA-based species-specific biosensors based on electrochemical impedance spectroscopy (EIS) for the detection of *P. falciparum*, *P. malariae* and *P. ovale* using micro-gold electrodes. EIS is a sensitive electrochemical technique that involves the application of a small amplitude sinusoidal voltage to measure electron blockage properties occurring at the surface of an electrode (Kanyong *et al*., 2020a). Unlike SERS, EIS enables reagent-free analysis that allows for the development of scalable diagnostic devices for ultrasensitive detection of target analytes (Kanyong *et al*., 2020a; Kanyong *et al*., 2020b; Kanyong & Davis, 2020). In addition, the micro platform of the gold electrode used in this study makes it economically feasible to mass produce the final device on a large scale for routine diagnosis of malaria. This study demonstrates the potential application of the biosensors for rapid, cost-effective and accurate species-specific diagnosis of malaria at the POC.

6.4 Materials and Methods

6.4.1 Materials and reagents

Sputtered chips consisting of micro-gold electrodes (µAuE) (Ø = ~700 µm) were purchased from FlexMedical Solutions (Scotland, UK). Chemical reagents used in the study include 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate ($NaHCO_3$), phosphate-buffered saline (PBS) tablets, 6-mercapto-1-hexanol (MCH), and potassium ferrocyanide ($K_4[Fe(CN)_6]$). All chemical reagents were of analytical grade and were purchased from Sigma Aldrich, UK. The Hellmanex III cleaning solution was purchased from Sigma Aldrich, UK. Milli-Q ultra-pure water was obtained from the Millipore Milli-Q Integral Water Purification System (Millipore Corporation, Massachusetts, USA). The detection probes and the oligonucleotides used in the study (**Table 9**) were purchased from Sigma Aldrich, UK.

Table 9: List of detection probes and oligonucleotides for biosensor development.

Target	DNA probe/Target sequence	Sequence
P. falciparum	Detection probe	5'-HS-$(CH_2)_6$-GTAACTATTCTAGGGGAACTATTTTAGC-3'
	Complementary	5'-GCTAAAATAGTTCCCCTAGAATAGTTAC-3'
	Three-base mismatch*	5'-GCT**G**AAATAGTTC**A**CCTAGAATAGT**G**AC-3'
P. malariae	Detection probe	5'-HS-$(CH_2)_6$-GTTGTACGTTAAGAATAACCGCCAAGGC-3'
	Complementary	5'-GCCTTGGCGGTTATTCTTAACGTACAAC-3'
	Three-base mismatch*	5'-GCC**C**TGGCGGTTAT**C**CTTAACGTAC**T**AC-3'
P. ovale	Detection probe	5'-HS-$(CH_2)_6$-GATGCTTAGACAATACAACGTATCTG-3'
	Complementary	5'-CAGATACGTTGTATTGTCTAAGCATC-3'
	Three-base mismatch*	5'-CAG**C**TACGTTG**A**ATTGTCTAAGC**T**TC-3'
	Non-complementary	5'-AACCCAAAGACTTTGATTTCTCATAA-3'

***The highlighted nucleotides (red) indicate mismatched bases

6.4.2 Characterisation of micro-gold electrode

Electrochemical experiments were carried out on the PGSTAT204 Autolab Potententat/Galvanostat/EIS FRAM32 Module (Metrohm-Atolab, Netherlands). The architecture of the sputtered chip consisting of the µAuE is illustrated in **Figure 14A**. To characterise the bare electrodes, the chips were washed in 30% Hellmanex III solution for 30 minutes at room temperature, rinsed three times in excess Milli-Q water, and dried under a gentle stream of nitrogen gas. Following the cleaning of the electrodes, differential pulse voltammetry (DPV), cyclic voltammetry (CV) and EIS analysis were performed in 5.0 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ prepared in 10 mM PBS (pH = 7.4). EIS response was carried out at 0.16 V over a frequency range of 100 kHz - 0.1 Hz.

6.4.3 Immobilisation of detection probes

The fabrication of the biosensors was carried out following previously described experimental procedures described (Butterworth *et al*., 2019; Keighley *et al*., 2008) with few modifications. Briefly, following the successful characterisation of the bare electrodes, the detection probes were self-assembled on the electrodes using 10 µL of the probe solution containing 1.0 µM thiol-modified species-specific detection probe and 1.0 mM MCH solution (10 mM PBS, pH = 7.4) and then incubated overnight at 4°C in a humidified chamber. Each of the species-specific modified electrodes was then rinsed in excess Milli-Q water to remove excess and/or unbound probes and MCH. EIS analysis was performed for the modified gold electrodes (Au/probe/MCH) in 5.0 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ as earlier described in this study. The *Plasmodium species*-specific modified electrodes were then rinsed in excess Milli-Q water and stored in sterile 10 mM PBS solution (pH = 7.4) at 4°C until ready for use. The sequences of the thiol-modified DNA detection probes for *P. falciparum*, *P. malariae* and *P. ovale* are shown in **Table 9.**

6.4.4 Analytical performance of biosensors

Using optimal conditions, the limit of blank (LoB), limit of detection (LOD), the sensitivity and the specificity of the *P. falciparum*, *P. malariae* and *P. ovale* biosensors were determined. The LOD and sensitivity were determined using oligonucleotides that are complementary to the detection probe (cDNA) for each of the three *Plasmodium* species (**Table S1**). Briefly, the species-specific cDNA oligonucleotides were serially diluted in nuclease-free water to concentrations in the range of 10 aM - 320 aM. A volume of 10 µL of each diluted cDNA concentration was incubated at 95 ℃ for 5 minutes and then applied to their corresponding modified electrodes (Au/probe/MCH) at 37 ℃ for 15 minutes. Following the incubation, the electrodes were washed in excess Milli-Q water and dried under a gentle stream of nitrogen gas, and EIS spectra acquired. The resulting spectra were analysed using bode plots. The normalized R_{ct} values (Relative Response, RR) of the biosensors were calculated using the equation $RR = [(R_1 - R_0)/ R_0]$, where R_0 and R_1 represent the charge transfer resistance before and after addition of the cDNA, respectively, obtained within the frequency range of 100 kHz - 0.1 Hz. The RR values were plotted against the $\log_{10}$-transformed concentrations of the cDNA to obtain a calibration plot from which the LOD and sensitivity were estimated. The LOD and sensitivity were determined using the formula $3.3 \times \sigma/S$ and S/A, respectively, where σ is the standard deviation of the blanks, S is the slope of the calibration curve, and A is the surface area of the µAuE. LoB for each of the biosensors was determined using 10 mM PBS solution (pH = 7.4). The limit of blank RR (RR_{LoB}) of the three biosensors were obtained using the formula $RR_{LoB} = \bar{x}_{Blank} + 3\sigma$, where $\bar{x}_{Blank}$ is the mean RR of the blanks and σ is the standard deviation of the blanks.

To determine the specificity, the species-specific modified electrodes (Au/probe/MCH) were independently incubated with 10 µL of 100 aM of the target cDNA, 3 base-pair mismatch and non-complementary oligonucleotides at 37 ℃ for 15 minutes. The resulting electrodes were

washed in excess Milli-Q water and dried under a gentle stream of nitrogen gas. EIS spectra were then acquired to determine the relative response (RR). The sequences of the cDNA, the 3 base-pair mismatch and the non-complementary oligonucleotides are shown in **Table 9**.

6.4.5 Species-specific detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical isolates

To validate the biosensors for the detection of *Plasmodium* parasites in clinical isolates, a total of 24 samples were selected from a large pool of cryopreserved clinical isolates that have been analysed using quantitative real-time polymerase chain reaction (qPCR) in our previous study (**Chapter 3**). The details of the qPCR assays have been described in our initial study (**Chapter 3**). The 24 samples that were selected based on the qPCR results consisted of twelve samples for *P. falciparum* assay (nine positives and three negatives), six samples for *P. malariae* assay (three positives and three negatives), and six samples for *P. ovale* assay (three positives and three negatives). Each of the 24 samples had purified genomic DNA and the paired whole blood samples.

For purified DNA samples, genomic DNA was purified from 200 µL of the whole blood using the QIAamp DNA Extraction Mini Kit (Qiagen, UK) and eluted with 100 µL elution buffer following the instructions from the manufacturer. The purified genomic DNA samples were incubated at 95 ℃ for 10 minutes and immediately placed on ice. The heating process enables denaturation of the double-stranded genomic DNA, while the ice limits immediate renaturation of the denatured genomic DNA. A volume of 10 µL of each of the denatured DNA samples was applied to the species-specific modified electrodes (Au/probe/MCH) and incubated at 37 ℃ for 15 minutes. The electrodes were then washed in excess Milli-Q water and then dried

under a gentle stream of nitrogen gas. Using the EIS analysis, the relative response (RR) of the biosensors were determined as described earlier in this study.

Lastly, the direct use of clinical samples without DNA pre-purification was investigated. To achieve this, a volume of 10 µL whole blood was diluted with 90 µL of lysis buffer (Qiagen, UK). The resulting whole blood lysate was incubated at 95 ℃ for 10 minutes and immediately placed on ice. A volume of 10 µL of the lysate was then applied to the modified electrodes (Au/probe/MCH) and incubated at 37 ℃ for 15 minutes. The electrodes were washed in excess Milli-Q water, dried under a gentle stream of nitrogen gas and then EIS spectra acquired to determine the relative response (RR).

6.5 Results

6.5.1 The principle of detection of parasite genomic DNA

DNA-based impedance biosensors were developed for the detection of *P. falciparum*, *P. malariae* and *P. ovale* using micro-gold electrodes. As shown in **Figure 14A**, each chip consists of an electrical connector, an insulating dielectric material, reference electrode, counter electrode, and a working micro-gold electrode (µAuE). A stepwise schematic representation of the assay development workflow is shown in **Figure 14B**. Firstly, the impedance spectra of the cleaned bare micro-gold (Au) electrodes were determined. Following this, the bare electrodes were incubated with the detection probe solution containing the thiolated probe (1.0 µM) and the MCH solution (1.0 mM). Co-immobilisation approach of the probe and MCH was used since this process produces a consistent self-assembled monolayer on an electrode (Butterworth *et al*., 2019; Keighley *et al*., 2008). The MCH was used to block any exposed surface on the electrode to minimise non-specific interactions. Following the modification, the impedance spectra of the modified electrodes (Au/probe/MCH) were determined. The modified electrodes were then incubated with the target DNA or sample of interest. The target DNA, if present, interacts with the immobilised probe, which is characterised by an increase in R_{ct} since the flow of electrons produced by the $[Fe(CN)_6]^{3-}$ /$[Fe(CN)_6]^{4-}$ redox couple is hindered at the surface of the electrode (Kanyong *et al*., 2020a). As a result, higher R_{ct} value is expected in the presence of the target DNA (Au/probe/MCH/DNA_{target}) compared to the R_{ct} value before the addition of the sample of interest (Au/probe/MCH). The resulting R_{ct} values are directly proportional to the concentrations of the captured target DNA. On the other hand, in the absence of the target DNA, no significant change in R_{ct} is expected before and after the addition of the sample of interest.

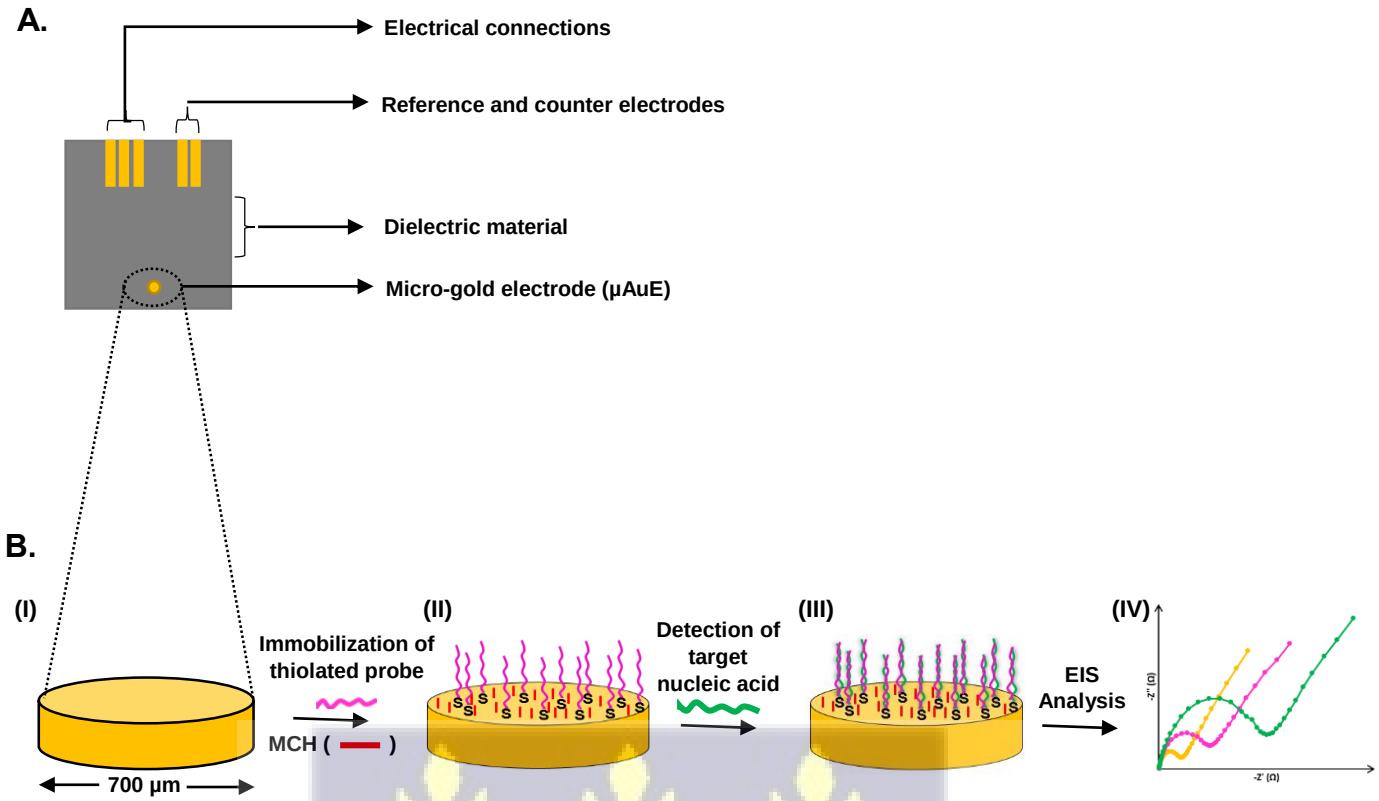

Figure 14: The architecture of the sputtered chip and the schematic representation of the workflow.

(A) The architecture of the chip consisting of an electrical connection section, reference electrode, counter electrode and an insulating dielectric material (ash) that defines the working area of the µAuE. **(B) (I)** Extended view of the working electrode (µAuE) with a diameter of ~700 µm. **(II)** The bare electrode was incubated with detection probe solution containing a mixture of the thiolated detection probe (magenta) and MCH blocking agent (red). **(III)** Incubation of the modified electrode with the target DNA (green). (**IV**) The resulting electrode was analysed using EIS. The expected Nyquist plots for the bare electrode (yellow), Au/probe/MCH electrode (magenta) and Au/probe/MCH/DNA_{target} electrode (green) following the stepwise modification. MCH = 6-mercapto-1-hexanol and EIS = electrochemical impedance spectroscopy.

6.5.2 Characterisation of the micro-gold electrode

DPV, CV and EIS were used to determine the electrochemical properties of the bare electrode. From the DPV analysis, the peak current and the corresponding potential for the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple were 2.27 ± 0.27 µA and 0.16 ± 0.01 V, respectively (**Figure 15A)**. The resulting average potential (0.16 V) was then used to acquire all the impedance spectra. Based on the CV analysis, an increase in current was observed with an increasing scan rate in the range of 10 mV/s - 300 mV/s (**Figure 15B**). Good linear relationships were obtained for both the peak anodic and peak cathodic currents with coefficient of determination, $R^2 > 0.99$ (**Figure 15C**). The R_{ct} values recorded on different bare µAuE indicated that the electrodes were highly reproducible with an average correlation coefficient of 96.6% ± 0.9% (**Figure 15D**).

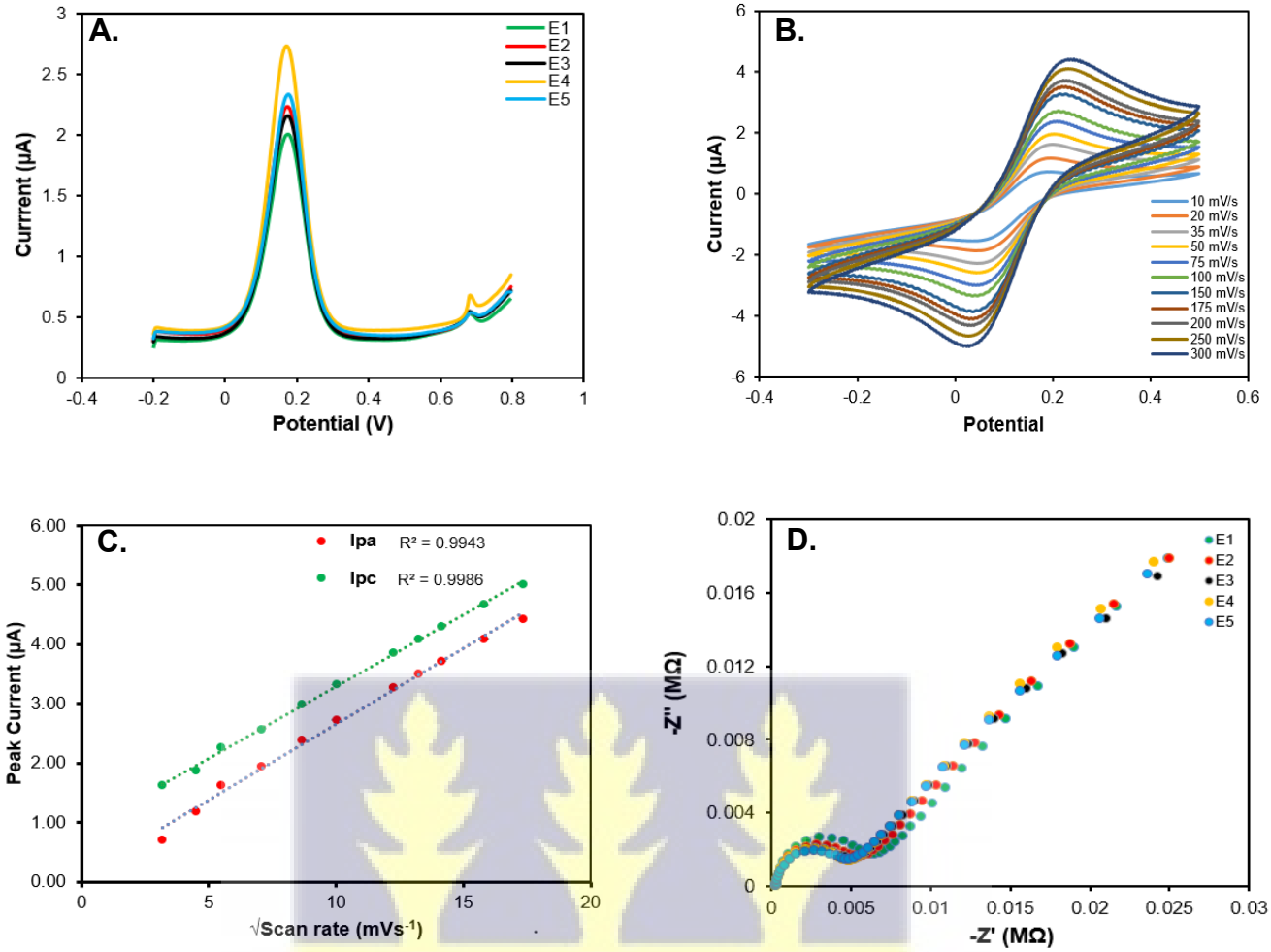

Figure 15: Characterisation of the bare micro-gold electrode (µAuE).

(**A**) DPVs for five different electrodes. (**B**) CVs for the bare electrode in 5.0 mM at different scan rates ranging from 10 mV/s to 300 mV/s. (**C**) Relationship between the peak anodic, Ipa (Red) and peak cathodic, Ipc (Green) currents as a function of the scan rate. A linear correlation was observed for both the peak anodic (Red) and peak cathodic (Indigo) currents with $R^2 > 0.99$. (**D**) Nyquist plots showing the charge transfer resistance (R_{ct}) of five independent electrodes. All measurements were recorded in 5.0 mM $[Fe(CN)_6]^{-3/-4}$ prepared in PBS (10 mM; pH 7.4).

6.5.3 Analytical performance of biosensors

The dose responses for the detection of *P. falciparum*, *P. malariae* and *P. ovale* are expressed in the Bode plots in **Figure 16A** with their corresponding calibration plots in **Figure 16B**. The sensitivity and the LOD of each species-specific biosensor were determined using cDNA concentrations in the range of 10 aM - 320 aM. Increasing impedance spectra were observed with increasing concentration of the cDNA for each of the three species-specific biosensors as shown in the bode plots in **Figure 16A**. There were good linear relationships between the relative response (RR) and the $\log_{10}$–transformed concentrations of the cDNA for all the three species-specific biosensors (**Figure 16B**). The fitted linear regression equations for the *P. falciparum*, *P. malariae* and *P. ovale* biosensors expressed as Relative Response (RR) were $RR = 3.2175*\text{Log}_{10}$ [cDNA] - 3.3425 ($R^2 = 0.9690$), $RR = 3.2378*\text{Log}_{10}$ [cDNA] - 3.5425 ($R^2 = 0.9443$), and $RR = 2.3837*\text{Log}_{10}$ [cDNA] - 2.1509 ($R^2 = 0.9697$), respectively. Based on the linear relationships, the sensitivities for the biosensors were 868.4 $M\Omega.aM^{-1}.cm^{-2}$, 920.4 $M\Omega.aM^{-1}.cm^{-2}$ and 558.8 $M\Omega.\ aM^{-1}.cm^{-2}$ for the *P. falciparum*, *P. malariae* and *P. ovale* biosensors, respectively (**Table 10**). The estimated LODs for the *P. falciparum*, *P. malariae* and *P. ovale* biosensors were 18.7 aM, 43.6 aM and 27.9 aM, respectively (**Table 10**). The RR for the limit of the blank (RR_{LoB}) obtained using sterile PBS for the three biosensors were $RR_{LoB} = 0.17$, $RR_{LoB} = 0.10$, and $RR_{LoB} = 0.28$ for *P. falciparum*, *P. malariae* and *P. ovale* biosensors, respectively (represented as short-dashed lines in **Figure 16B**). The LODs of these biosensors are comparable to some previously reported DNA-based sensors, which were also in the attomolar range (Kaur *et al*., 2018; Li *et al*., 2016; Rahman *et al*., 2017). To the best of our knowledge, this study describes the first species-specific biosensors for the detection of *P. malariae* and *P. ovale* parasites. The observed LODs of the current biosensors are exceptionally low for an unamplified label-free analysis. These characteristics are notably superior, being the first impedance-based sensors for species-specific malaria diagnosis in clinical samples. This

electrochemical platform, unlike those highlighted in **Table 10**, is also single-step and require no pre-amplification step; which could be readily integrated into both automated fluid handlers and multiplex formats with retention of assay specificity. In addition, the use of synthetic nucleic acid detection probes would make the biosensors relatively less expensive for population-wide surveillance and routine POC diagnosis of malaria. Cost is a relevant point to be considered for the commercialisation of these devices especially in resource-limited malaria endemic settings such as sub-Saharan Africa.

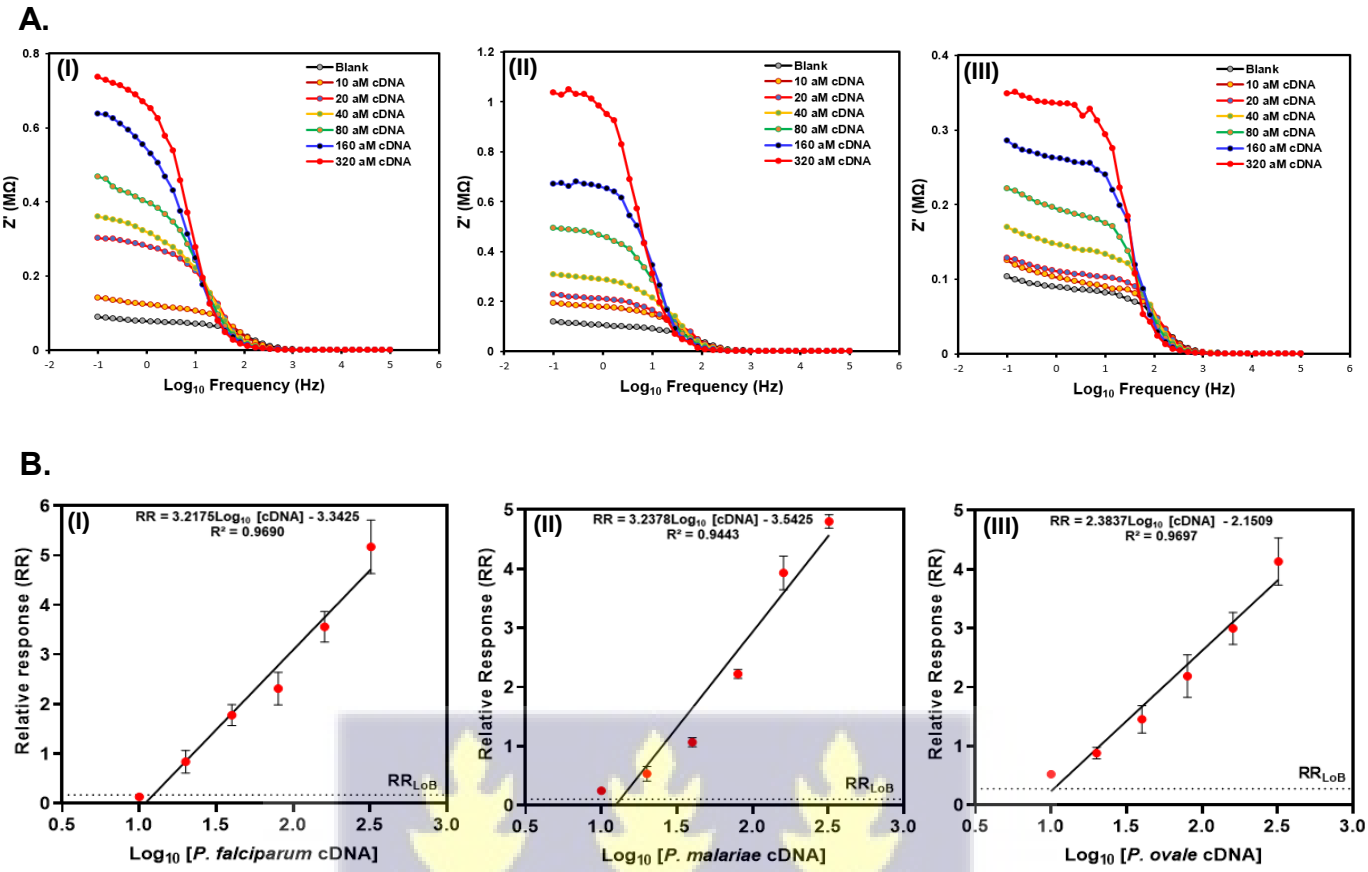

Figure 16: The sensitivity of the biosensors.

(A) Bode plots for *P. falciparum* **(I)**, *P. malariae* **(II)** and *P. ovale* **(III)** biosensors with cDNA concentrations ranging from 10.0 aM to 320.0 aM. Increasing impedance (Z') were observed with increasing cDNA concentration for all three *Plasmodium* species. **(B)** The linear relationship between the relative response (RR) and the $\log_{10}$-transformed cDNA concentrations for *P. falciparum* **(I)**, *P. malariae* **(II)** and *P. ovale* **(III)** obtained from their corresponding bode plots (n = 3). The impedance spectra for determining the RR were acquired within the frequency range of 100 kHz - 0.1 Hz. Short-dashed lines represent limit of blanks (LoB).

Table 10: Comparison of the LODs of DNA-based biosensors for the detection of *Plasmodium* species.

Target	Nanomaterial	Detection technique	Initial pre-amplification	LOD	Sensitivity ($\mu\Omega.aM^{-1}.cm^{-2}$)	Reference
P. falciparum	Gold	QCM*	PCR	0.025 ng/mL	-	(Potipitak *et al.*, 2011)
P. falciparum	Silver	QCM*	PCR	-	-	(Wangmaung *et al.*, 2014)
P. vivax	Silver	QCM*	PCR	-	-	(Wangmaung *et al.*, 2014)
P. falciparum	Magnetic beads and nano-rattles	SERS#	Not required	100 aM	-	(Ngo *et al.*, 2016)
P. falciparum	Gold	EIS	Not required	18.7 aM	868.4	Current study
P. malariae	Gold	EIS	Not required	43.6 aM	920.4	Current study
P. ovale	Gold	EIS	Not required	27.9 aM	558.8	Current study

LOD = Limit of detection, QCM* = Quartz crystal microbalance; SERS# = Surface-enhanced Raman scattering, EIS = Electrochemical impedance spectroscopy, and "– " means value not reported.

For specificity, the relative response (RR) for the cDNA oligonucleotides for all the three species-specific biosensors were at least 4-fold higher than that of the non-complementary and the 3-base pair mismatch oligonucleotides (**Figure 17**). The recovery rate of target cDNA was also assessed by spiking *Plasmodium*-negative whole blood sample with the target cDNA. The cDNA recovery rates (%) for the spiked lysates were 85.4%, 87.8% and 78.1% for the *P. falciparum*, *P. malariae* and *P. ovale* biosensors, respectively (**Table 11**). The Relative Standard Deviation (RSD) recorded for the three biosensors ranged from 2.06% to 8.16%

(**Table 11**). A decrease in the relative response was observed for all the three species-specific biosensors when incubated with the unspiked *Plasmodium*-negative whole blood lysate (**Table 11**). This observation could be due to the electrical properties of blood and its constituents including haemoglobin and electrolytes (e.g. sodium and potassium ions) which could interact with the negatively charged phosphodiester backbone of the immobilised DNA probe (Rahman *et al*., 2017).

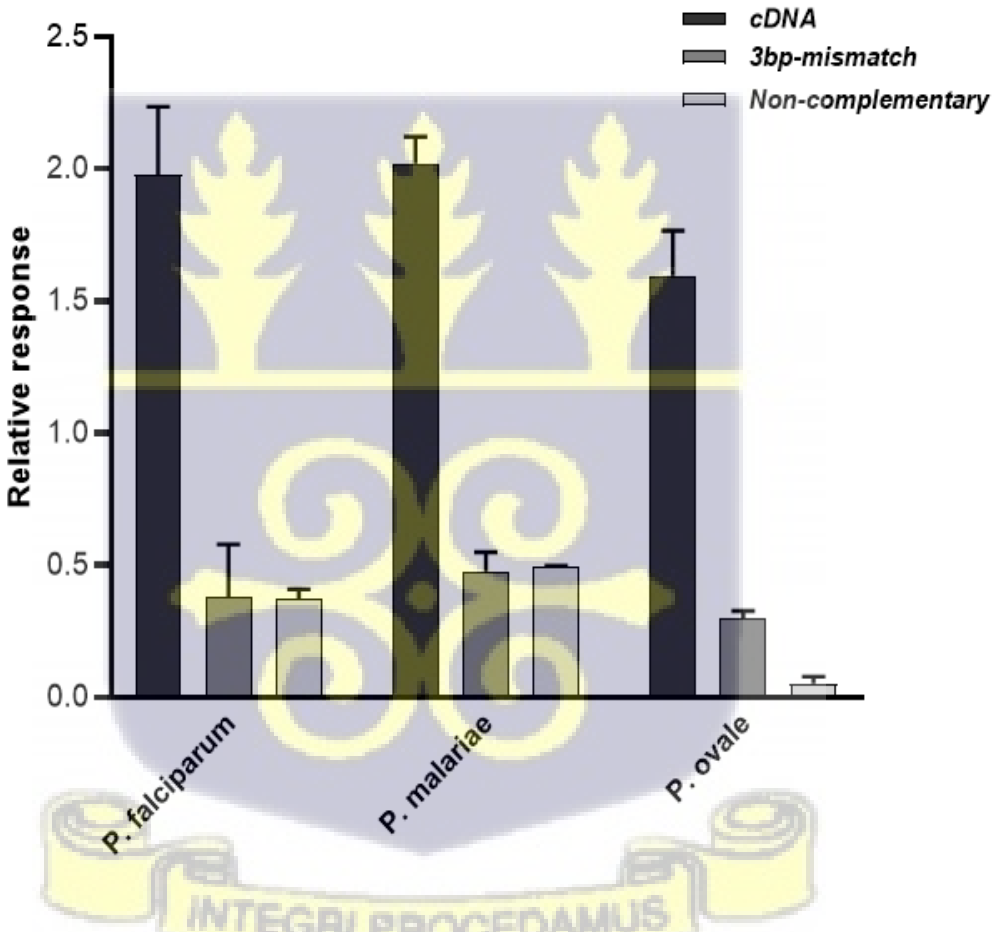

Figure 17: The specificity of the biosensors.

The specificity of *P. falciparum*, *P. malariae*, and *P. ovale* biosensors using complementary (cDNA), 3 base-pair (bp) mismatch and non-complementary oligonucleotides.

Table 11: cDNA-recovery from whole blood lysates spiked with 100 aM of target cDNA.

Biosensor	*Relative response (RR)			Recovery rate	#Recovery	RSD
	cDNA only	cDNA-spiked lysate	Unspiked lysate	(%)	(aM)	(%)
P. falciparum	1.98 ± 0.26	1.69 ± 0.16	- 0.41 ± 0.02	85.4	85.4	8.16
P. malariae	2.05 ± 0.12	1.80 ± 0.08	- 0.94 ± 0.06	87.8	87.8	7.65
P. ovale	1.60 ± 0.17	1.25 ± 0.10	- 0.49 ± 0.06	78.1	78.1	2.06

*The normalised relative response (RR) values are presented as average from replicates plus or minus standard deviation (n = 3). #Recovery represents concentration of cDNA recovered from the lysates spiked with 100aM of target cDNA. RSD = relative standard deviation.

Table 12: The diagnostic performance of the biosensors compared to qPCR assays.

Biosensor	Sample size	Sample type	Sensitivity (%)	Specificity (%)
P. falciparum	12	Purified genomic DNA	100	66.7
		Whole blood lysate	22.2	100
P. malariae	6	Purified genomic DNA	100	100
		Whole blood lysate	33.3	100
P. ovale	6	Purified genomic DNA	100	66.7
		Whole blood lysate	66.7	100

6.5.4 Detection of *Plasmodium* species genomic DNA in clinical isolates

The practicability of the biosensors for the detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical isolates was assessed using purified genomic DNA samples and the paired whole blood lysates that were selected from a previously qPCR-analysed sample pool **(Chapter 3)**. Positivity was defined by an increase in the relative response (RR) following incubation of the modified electrode with the sample of interest. To identify positive samples, the limit of blank RR (RR_{LoB}) was used as the baseline or threshold values for each of the three *Plasmodium* species. Clinical samples with RR above the threshold values were considered positive. The diagnostic sensitivity and specificity of the biosensors were determined as described earlier in **section 5.4.7**. Compared to the qPCR assays, the diagnostic sensitivity of the *P. falciparum*, *P. malariae* and *P. ovale* biosensors using purified genomic DNA samples were 100% (9/9), 100% (3/3) and 100% (3/3), respectively (**Figures 18A - 18C**; **Table 12**). The diagnostic specificity of the *P. falciparum*, *P. malariae* and *P. ovale* biosensors for the purified genomic DNA samples were 66.7% (2/3), 100% (3/3) and 66.7% (2/3), respectively (**Figures 18A - 18C**; **Table 12**). Using the paired whole blood lysates, the diagnostic sensitivity of the *P. falciparum*, *P. malariae* and *P. ovale* biosensors were 22.2% (2/9), 33.3% (1/3) and 66.7% (2/3), respectively (**Figures 18A - 18C**; **Table 12**). Also, the specificity for the whole blood lysates were 100% (3/3) for the three *Plasmodium* species-specific biosensors (**Figures 18A - 18C**; **Table 12**).

In redefining the threshold line using the RR of the non-specific (non-complementary) oligonucleotide ($RR_{Non\text{-}specific}$) for *P. falciparum* (0.37), *P. malariae* (0.50) and *P. ovale* (0.05) as shown in **Figure 18**, the purified DNA samples had the same diagnostic sensitivity (100%) and specificity (66.7 – 100%) (**Figure 18A - 18C**). Similarly, the diagnostic performance obtained for the *P. falciparum* and *P. ovale* biosensors on the whole blood lysates using the RR of the non-complementary oligonucleotide as the threshold line were comparable to the

diagnostic performance obtained with the RR_{LoB} as the threshold (**Figure 18A and 18C**). The *P. malariae* biosensor, however, had reduced sensitivity of 0% (0/3) as the RR of the qPCR-positive samples were below the RR of the non-complementary oligonucleotide (**Figure 18B**). It is important to highlight that the limited sample size used in this study may not reflect the actual diagnostic performance of the biosensors. As such, further studies involving a larger clinical sample size will be necessary to properly estimate the diagnostic performance of the biosensors relative to other established methods such as PCR.

We finally assessed parasite quantification using the biosensors by correlating the relative response (RR) with the qPCR threshold cycle (C_t) values (**Figure 18D**). The RR values moderately correlated with the qPCR C_t-values with coefficient of determination, $R^2 = 0.425$ for purified genomic DNA and $R^2 = 0.266$ for whole blood lysates, indicating that the biosensors have the potential to quantitatively detect *Plasmodium* species in both purified DNA samples and whole blood lysates.

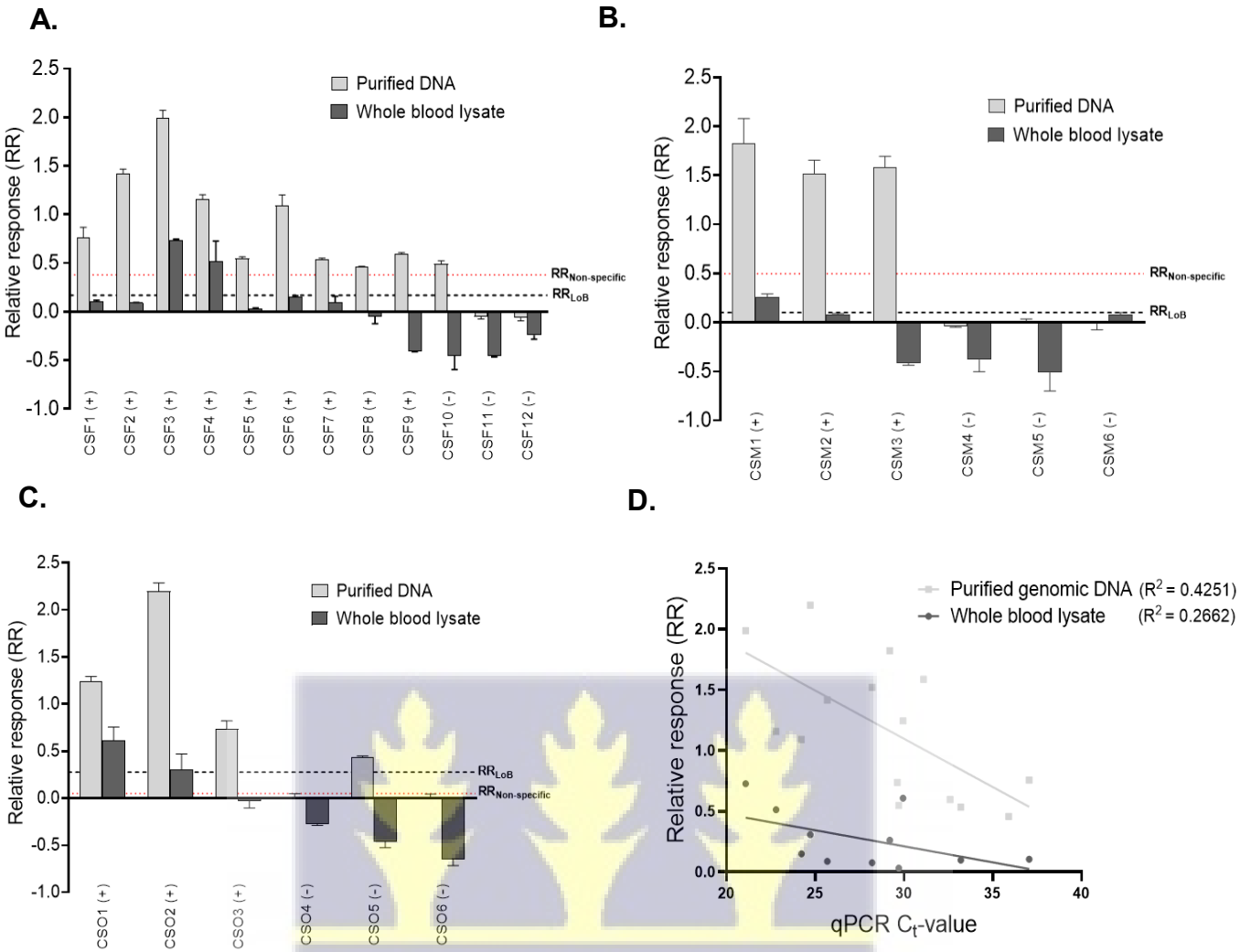

Figure 18: Detection of *Plasmodium* species in purified genomic DNA and whole blood lysates obtained from clinical isolates.

(A) Relative response for *P. falciparum* biosensor for twelve clinical samples (CSF1 - CSF12). **(B)** Relative response for *P. malariae* biosensor for six clinical samples (CSM1 – CSM6). **(C)** Relative response for *P. ovale* biosensor for six clinical samples (CSO1 – CSO6). The symbols "+" and "-" represent qPCR results for positive and negative samples, respectively. Short-dashed lines labelled RR_{LoB} (Black) and $RR_{Non\text{-}specific}$ (Red) represent the RR for the limit of the blank and non-specific oligonucleotide, respectively. The short-dashed lines were used as threshold values for determining positivity. **(D)** Correlation analysis between qPCR C_t-values and biosensor relative response. R^2 represents the coefficient of determination, (n = 3).

6.6 Discussion and conclusion

P. malariae and *P. ovale* are usually detected as low-density infections below the limits of detection of the current POC malaria diagnostic tools (Berhane *et al*., 2018; Cunningham *et al*., 2019; Moody, 2002; Wongsrichanalai *et al*., 2007). In addition, cases of submicroscopic *P. falciparum* have been reported (Lo *et al*., 2015; Walker-Abbey *et al*., 2005). To achieve adequate diagnostic sensitivity, an initial amplification of target DNA is generally required for the detection of these low-density infections (Potipitak *et al*., 2011; Wangmaung *et al*., 2014). This pre-amplification step usually requires a time-consuming nucleic acid purification stage, expensive reagents and is also susceptible to cross-contamination, which renders the process less suitable to routine POC diagnosis. In this study, we developed DNA-based label-free electrochemical biosensors for species-specific detection of *P. falciparum*, *P. malariae* and *P. ovale* in clinical isolates without the need for initial amplification of the target DNA. This characteristic of the current biosensors offers several advantages, including the elimination of well-known challenges and risks associated with a pre-amplification step.

The LOD of the current *P. falciparum* biosensor is approximately 5-fold lower compared to a previously reported *P. falciparum* SERS-based DNA biosensor, which also does not require pre-amplification (Ngo *et al*., 2016). To the best of our knowledge, this study describes the first DNA-based biosensors for the detection of *P. malariae* and *P. ovale* parasites with limits of detection lower than the previously reported SERS-based DNA biosensor (Ngo *et al*., 2016). The observed low LODs of the current biosensors could be due to the micro-size of the gold electrodes and the EIS detection mechanism used in this study. The low LODs of the biosensors will be essential for the detection of low-density falciparum and non-falciparum infections. In addition, the reagent-free nature of the EIS-based system and the use of synthetic nucleic acid detection probes would make the biosensors relatively economical for routine POC diagnosis (Kanyong *et al*., 2020a).

Notably, all the three species-specific biosensors had a diagnostic sensitivity of 100% using the purified genomic DNA compared to the qPCR assays. Although the sensitivities reduced to the range of 22.2% - 66.7% using the whole blood lysate, these are higher compared to microscopy (Berzosa *et al*., 2018; Mehlotra *et al*., 2000; Mfuh *et al*., 2019; Snounou *et al*., 1993a; Walker-Abbey *et al*., 2005) and RDTs (Charpentier *et al*., 2020) as reported for detection of low-density *Plasmodium* infections.

In conclusion, the results presented show that the DNA-based impedance biosensors can detect *P. falciparum*, *P. malariae* and *P. ovale* with high sensitivity and specificity. More significantly, the biosensors could be used to detect *Plasmodium* species in clinical isolates without initial amplification of target DNA. In addition, the results could be obtained within 30 minutes which is comparable to the readout time of microscopy and RDTs, but lower than the turnaround time for nucleic acid-based amplification assays such as PCR and LAMP.

CHAPTER SEVEN

7.0 General Discussion, Conclusion, and Recommendations for Future Work

7.1 General Discussion

P. malariae and *P. ovale* infections are generally known to cause milder malaria; however, recent studies have associated these less prevalent non-falciparum species in major disease burden such as severe anaemia and kidney-related complications (Rojo-Marcos *et al.*, 2008; Douglas *et al.*, 2013; Silva *et al.*, 2017; D'Abramo *et al.*, 2018; Gentile *et al.*, 2019; Kotepui *et al.*, 2020a). In addition, there are reports of increasing prevalence of these non-falciparum species in settings with reduced *P. falciparum* transmission (Betson *et al.*, 2018; Gnémé *et al.*, 2013; Yman *et al.*, 2019). These reports necessitate the availability of reliable assays for species-specific detection of *P. malariae* and *P. ovale*. However, the most readily available and cost-effective detection tools, including microscopy and antigen-based RDTs, lack adequate sensitivity and specificity for species-specific detection of *P. malariae* and *P. ovale* in clinical isolates (Gimenez *et al.*, 2021).

Currently, PCR-based assays are generally considered the most reliable method for the detection of *P. malariae* and *P. ovale* (Gimenez *et al.*, 2021). Despite the characteristic high sensitivity and specificity of PCR-based assays, accurate detection of low-density *Plasmodium* infections remains a major challenge (Hofmann *et al.*, 2015). One of the major factors known to limit the sensitivity and specificity of PCR-based assays is the formation of non-specific amplicons such as primer-dimers (Chou *et al.*, 1992; Satterfield, 2014). Over the years, several approaches, including the use of hot-start reagents and modified primers, have been developed to prevent or limit the formation of non-specific products (Barnes & Rowlyk, 2002; Lebedev *et al.*, 2008; Satterfield, 2014; Satterfield *et al.*, 2007). Among these, cooperative primers were the first technology shown to curb the formation and propagation of non-specific products

(Poritz & Ririe, 2014; Satterfield, 2014). Cooperative primer-based assays have been shown to have lower detection limits than assays involving the use of the corresponding conventional primers (Satterfield, 2014). In this study, cooperative primer-based qPCR assays were developed for the detection of *P. malariae* and *P. ovale* in field samples.

The data presented here indicate that the cooperative primer-based qPCR assays had at least a 10-fold lower detection limit than the corresponding conventional primer-based qPCR assays. As expected, separation of the resulting qPCR amplicons on agarose gel revealed primer-dimers at lower concentrations for the conventional primer-based assays. However, no observable primer-dimers were seen for both the *P. malariae* and *P. ovale* cooperative primer-based qPCR assays. This observation parallels the finding in a previous study in which low copy numbers of template DNA that can be successfully amplified with cooperative primers were false-negatives using conventional primers due to primer-dimer background (Satterfield, 2014). The observed lower limits of detection of the cooperative primer-based qPCR assays described in this study may be explained by the ability of the cooperative primers to limit primer-dimer formation and propagation (Poritz & Ririe, 2014; Satterfield, 2014).

In Ghana, *P. malariae* and *P. ovale* are the non-falciparum species that have been implicated in clinical malaria (Amoah *et al*., 2019; DFID, 2011; Dinko *et al*., 2013; Owusu *et al*., 2017). However, due to the absence of reliable detection tools for the surveillance of these non-falciparum species, there is limited data on the distribution of *P. malariae* and *P. ovale*. As such, the newly cooperative primer-based qPCR assays were used in a cross-sectional study to determine the prevalence of *P. malariae* and *P. ovale* among study participants from three malaria transmission settings in Ghana. The overall prevalence of *P. malariae* and *P. ovale* among the combined study population were 13.3% and 4.8%, respectively. The prevalence of *P. malariae* and *P. ovale* reported in this study are higher compared to the estimated national prevalence of <10% and <2% for *P. malariae* and *P. ovale*, respectively (DFID, 2011).

Variation in the distribution of *P. malaria* and *P. ovale* has been reported among different populations and across different sites (Mueller *et al*., 2007). In this study, the frequency distribution of *P. malariae* was significantly higher among symptomatic participants than asymptomatic participants; however, *P. ovale* infection was comparable between the two groups. Contrary to these findings, a previous study in Indonesia observed a higher prevalence of non-falciparum species among asymptomatic participants than symptomatic participants (Karyana *et al*., 2008). Notably, a recent meta-analysis study comprising 20-year period data (2000 – 2020) also observed that the distribution of *P. malariae* and *P. ovale* between symptomatic and asymptomatic individuals was comparable (Hawadak *et al*., 2021). Also, age-dependent malaria immunity is known to affect the dynamics of *Plasmodium* species infections (Aponte *et al*., 2007). In this study, while the prevalence of *P. falciparum* was found to be decreasing with increasing age, both *P. malariae* and *P. ovale* prevalence were observed to be increasing with increasing age among symptomatic participants, which is consistent with previous studies in Burkina Faso (Gnémé *et al*., 2013) and Uganda (Betson *et al*., 2018). In addition, *P. malariae* was found to be most common among participants aged 11 – 20 years, while *P. ovale* prevalence was similar across the different age groups, which are in agreement with the findings in a previous study conducted elsewhere in Ghana (Ehrhardt *et al*., 2006). On the contrary, the study in Indonesia observed that the older population with a median age of 21 years had higher *P. malariae* infections compared to the younger population (Karyana *et al*., 2008). These differences in the distribution of non-falciparum species among different age groups could be due to the underrepresentation of the different age groups and differences in malaria transmission intensity across different sites (Lo *et al*., 2015, 2017). Taken together, these observations suggest differences in the transmission dynamics of *P. malariae* and *P. ovale* among different populations.

Anaemia is one of the major clinical symptoms associated with non-falciparum malaria (Douglas *et al*., 2013; Ehrhardt *et al*., 2006). The current study observed that participants with *P. malariae* and *P. ovale* infections had lower haemoglobin levels compared to participants without *Plasmodium* infection, and this observation parallels previous reports (Maina *et al*., 2010; Olliaro *et al*., 2011). Based on multivariate logistic regression analysis, mono-infections of *P. falciparum*, *P. malariae* and *P. ovale* were not significantly associated with the risk of developing mild or moderate anaemia. However, participants harbouring mixed infections of *P. falciparum* with either *P. malariae* or *P. ovale* had ~2-fold increased risk of developing mild or moderate anaemia. This finding corroborates previous studies, which observed that individuals with mixed infection of *Plasmodium* species had an increased risk of developing anaemia compared to those with mono-infection of *Plasmodium* species (Douglas *et al*., 2013; Ehrhardt *et al*., 2006). Taken together, the data suggest that interactions between falciparum and non-falciparum *Plasmodium* species may enhance malaria severity (Douglas *et al*., 2013; Maitland *et al*., 1996; Maitland *et al*., 1997; Mayxay *et al*., 2004; Price *et al*., 2001). As such, species-specific diagnosis of *P. malariae* and *P. ovale* at the POC will be necessary to inform the recommendation of appropriate antimalarial treatment and effective disease management.

Currently, the diagnosis of malaria at the POC primarily relies on microscopy and antigen-based RDTs, which lack adequate sensitivity and specificity for the detection of *P. malariae* and *P. ovale* (Cunningham *et al*., 2019; Gimenez *et al*., 2021; World Health Organisation, 2020). As such, POC diagnosis of malaria requires the application of sensitive and specific nucleic acid-based amplification tests (NAATs) such as PCR. However, the routine use of NAATs at the POC has been hampered by the high operational cost and the need for high technical expertise (LaBarre *et al*., 2011; Mueller *et al*., 2007). Therefore, in-house LAMP assays were developed in this study for cost-effective detection of *P. falciparum*, *P. malariae* and *P. ovale* using locally produced *Bst*-LF polymerase. Compared to PCR, LAMP is a

relatively quick and simple field-adaptable technique that requires minimal operational expertise (Becherer *et al*., 2020; Thompson & Lei, 2020). In comparison to the cooperative primer-based qPCR assays, the diagnostic sensitivity of the *P. falciparum*, *P. malariae* and *P. ovale* in-house RT-LAMP assays were 95.2%, 96.8% and 97.5%, respectively. Also, the diagnostic specificity were 90.0%, 86.2% and 87.3% for *P. falciparum*, *P. malariae* and *P. ovale* in-house RT-LAMP assays, respectively. In a recent systematic review and meta-analysis study comprising of LAMP assays that have been reported for the detection of *Plasmodium* species, the sensitivity and the specificity of LAMP compared to PCR were in the range of 80.0% - 98.8% and 86.5% - 98.8%, respectively (Picot *et al*., 2020). The analytical performance of the in-house LAMP assays described in this study are within the range of the reported LAMP assays. This development represents a major step towards local fabrication of LAMP kit for cost-effective nucleic acid-based diagnosis of both falciparum and non-falciparum malaria at the POC.

Alternative to the LAMP-based assays, the recent surge in demand for reliable POC diagnostic tools has drawn significant attention towards biosensing-based diagnostic devices (Krampa *et al*., 2020; Mehrotra, 2016; Rodovalho *et al*., 2015). A biosensor is a field-adaptable device that offers reliable, cost-effective and quick diagnosis (Mehrotra, 2016; Mohankumar *et al*., 2021). This study developed the first label-free DNA-based electrochemical biosensors for species-specific detection of *P. falciparum*, *P. malariae* and *P. ovale*. The LODs of the current DNA-based biosensors were lower than previous DNA-based biosensors reported for the detection of *Plasmodium* species (Ngo *et al*., 2016), *Salmonella spp.* (Berdat *et al*., 2007; García *et al*., 2012) and *Staphylococcus aureus* (Koydemir *et al*., 2014; Li *et al*., 2017). In addition to the improved sensitivity, the reagent-free nature of the EIS-based detection mechanism and the use of synthetic oligonucleotide biorecognition receptors would likely make the current biosensors relatively economical for routine POC application and population-wide diagnosis (Kanyong *et*

al., 2020a). The analytical performance of the *P. falciparum*, *P. malariae* and *P. ovale* biosensors using clinical isolates were compared to the qPCR assays. The biosensors had diagnostic sensitivity and specificity ranging from 22.2% to 100%. These observed sensitivities and specificities of the current biosensors are higher than that of microscopy (Berzosa *et al*., 2018; Mehlotra *et al*., 2000; Mfuh *et al*., 2019; Snounou *et al*., 1993a) and RDTs (Charpentier *et al*., 2020) for the detection of low-density *Plasmodium* infections.

7.2 Conclusion

In summary, the study developed DNA-based assays with high sensitivity and specificity for molecular surveillance and POC diagnosis of *P. malariae* and *P. ovale*. Previous studies on the surveillance of *Plasmodium* species have mainly focused on *P. falciparum* and *P. vivax*, which is partly due to the limited sensitivity and specificity of available assays for the detection of *P. malariae* and *P. ovale*. The data presented here show that the new cooperative primer-based qPCR assays described in this study (**Chapter 3**) have adequate sensitivity and specificity for the detection of *P. malariae* and *P. ovale*. The use of the new qPCR assays for routine surveillance of *P. malariae* and *P. ovale* would be important for properly assessing the distribution of these non-falciparum species. This is particularly important as data presented in this study (**Chapter 4**) indicate that mixed infections of these non-falciparum species are associated with an increased risk of developing malarial anaemia. More importantly, the routine species-specific diagnosis of *P. malariae* and *P. ovale* at the POC would be necessary for effective disease treatment and management. The in-house LAMP assays described in this study (**Chapter 5**) represent a major step towards the development of cost-effective, sensitive and specific nucleic acid-based amplification assays for species-specific diagnosis of *P. malariae* and *P. ovale* at the POC. In addition, the DNA-based electrochemical biosensors developed in this study (**Chapter 6**) are alternative diagnostic tools for timely and accurate species-specific detection of non-falciparum species. This study highlights the importance of integrating *P. malariae* and *P. ovale* detection tools into global malaria interventions and control programs towards a holistic approach to human malaria elimination.

7.3 Recommendations for Future Work

The study describes cooperative primer-based qPCR assays with improved analytical performance for the detection of *P. malariae* and *P. ovale*. However, the high cost of qPCR reagents could limit routine population-wide surveillance of these non-falciparum species. As such, further study using locally produced *Thermus aquaticus* (*Taq*) polymerase for the development of the in-house cooperative primer-based qPCR assays would be important for cost-effective surveillance of non-falciparum species.

The study also developed in-house SYBR Green-based RT-LAMP assays for the detection of *P. malariae* and *P. ovale*. Future study to develop colorimetric-based LAMP assays is recommended for a more simplified and economical diagnosis of non-falciparum malaria at the POC. Further to this, the deployment of the LAMP assays into clinical laboratories will help identify the challenges towards streaming for pilot implementation testing.

Lastly, future studies for further developing the current DNA-based biosensors into a multielectrode array (**Figure 19**) for the simultaneous detection of falciparum and non-falciparum *Plasmodium* species would be necessary for a more comprehensive diagnosis of malaria at the POC.

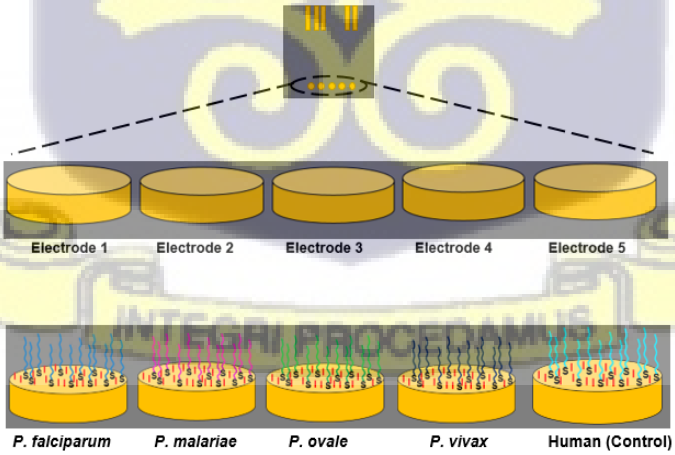

Figure 19: The proposed architecture of a multielectrode arrays biosensor for simultaneous detection of *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*.

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APPENDIX A: PRIMER-DIMERS AND COOPERATIVE PRIMERS

Appendix A-I: The formation of primer-dimers

The formation of primer-dimers is initiated by a random collision between a forward primer and a reverse primer (**Figure 20**). The probability of this collision between the primers is affected by several factors, including primer complementarity, primer melting temperature and primer concentration. In the event of interaction between the forward primer (green) and the reverse primer (blue), the polymerase extends the primers from the 3'-OH ends to produce amplicons that are complementary (**Figure 20**). The resulting amplicons are exponentially amplified during subsequent cycles to producing a detectable level of primer-dimers. This process interferes with the amplification of the target of interest, leading to false negatives or false positives.

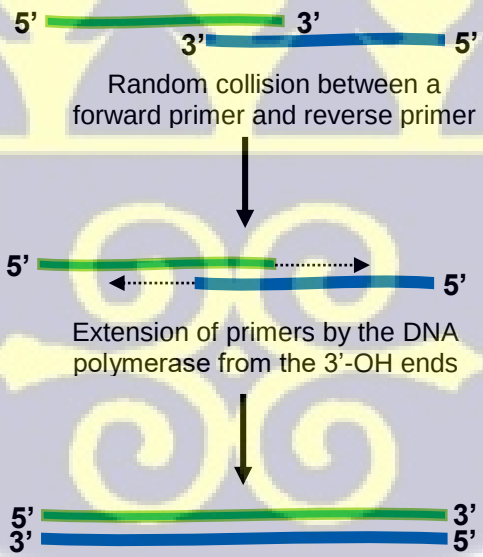

Figure 20: The formation of primer-dimers.

The extension of primers following a random collision between a forward primer (green) and a reverse primer (blue) results in primer-dimers

Appendix A-II: The structural composition of the cooperative primer

The structural composition of the cooperative is shown in **Figure 21**. The cooperative primer comprises of two stretches of oligonucleotides (a capture sequence and a low Tm primer) separated by a flexible non-extendable polyethylene glycol linker. The capture sequence, which binds few base pairs downstream low Tm primer, enables the low Tm primer to bind to its complementary target by bringing it in close proximity and thus increases the effective concentration by several orders of magnitude. This increases the chances of interaction between the low Tm primer and the target sequence since the low Tm primer by itself would not anneal at the selected annealing temperature for the amplification reaction.

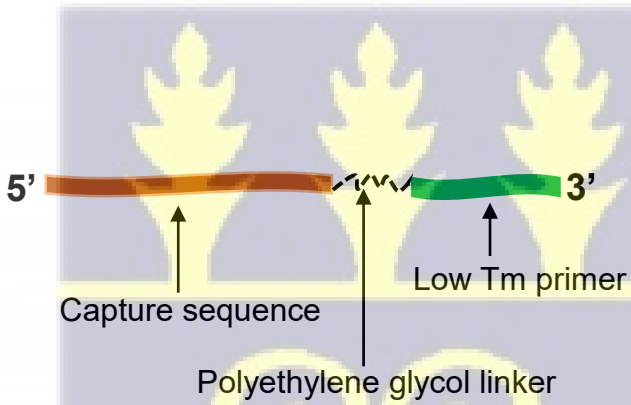

Figure 21: The structure of a cooperative primer.

The composition of cooperative primer showing the capture sequence (chocolate), the flexible polyethylene glycol linker (black) and the low Tm primer (green).

Appendix A-III: The process of amplification of the cooperative primer

The annealing and extension of cooperative primers during amplification as developed in our cooperative primer-based assays (**Chapter 3**) have been illustrated in 4 steps in **Figure 22**. Both the *P. malariae* and *the P. ovale* cooperative primer-based assays consisted of a forward cooperative primer and a paired conventional primer.

Step 1: During PCR, the capture sequence of the cooperative primer first binds to its complementary sequence.

Step 2: The binding of the cooperative sequence positions the low Tm primer in close proximity to its complementary sequence, thus allowing it to anneal.

Step 3: The PCR polymerase (yellow) binds and extends the low Tm primer at its free 3'–OH end.

Step 4: The downstream capture sequence of the cooperative primer is cleaved by the 5' – 3' exonuclease activity of the PCR polymerase during extension to produce the new amplicon.

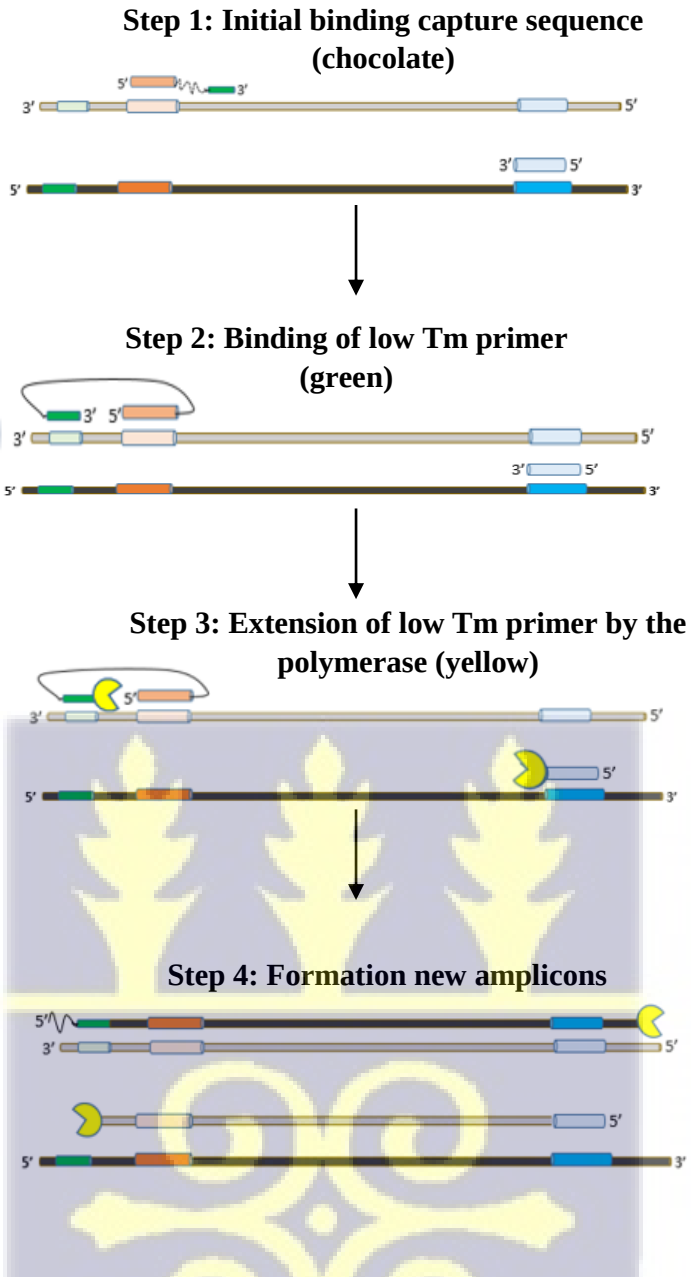

Figure 22: The process of annealing and extension of the cooperative primer.

The *P. malariae* and *P. ovale* assays consisted of a forward cooperative primer and a paired conventional primer. The process of annealing and extension of the cooperative primer has been illustrated.

Appendix A-IV: How cooperative primers limit the formation of primer-dimers

The two important factors that drive primer-dimer formation are random association between primers and the availability of extendable 3'-OH end (**Figure 20**). The structural composition of the cooperative primer (**Figure 21**) enables it to overcome these factors. This is achieved by limiting random collision between primers using a low Tm primer and the elimination of the 3'-OH group on the capture sequence using a non-extendable polyethylene glycol linker.

The low Tm primer of the cooperative primer usually has a melting temperature below the selected annealing temperature for the amplification of the target of interest. This low Tm limits interaction between the forward and reverse low Tm primers of the cooperative primers. The capture sequence, with higher Tm that is usually comparable to a convention primer, lack 3'-OH. As such, in the event of random collision capture sequences of the cooperative primers, the absence of 3'-OH limits the polymerase from extending the primer to produce primer-dimers.

APPENDIX B: ETHICAL APPROVAL FORMS

CONSENT FORM FOR ADULTS (18 YEARS AND ABOVE)

Title: The effects of Artemisinin-based combination therapy (ACT) on the dynamics of *Plasmodium falciparum, P. malariae* and *P. ovale* infections in Ghana

Principal Investigator: Prof Gordon A. Awandare

Co-investigators: Felix Ansah (student)

Dr Yaw Aniweh

Dr Emmanuel Amlabu

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P. O. Box LG54, Legon, Accra. Telephone: 0543 717 697. Email: gawandare@ug.edu.gh

General Information about Research

We are inviting you to participate in a research study that seeks to determine the distribution of malaria germs in Ghana and how these germs respond to the currently used artemisinin-based combination therapies (ACTs) administered in hospitals. We stopped using some malaria drugs such as chloroquine some years ago because they were no longer killing the malaria germs. Now, ACTs are the best malaria drugs we have. It has been reported in some parts of the world that the ACTs are also failing to kill some of the malaria germs. Also, there are different malaria germs, and each has a different response to the ACTs. Therefore, it is important to investigate the different malaria germs circulating in Ghana and see if the ACTs given at the hospitals in Ghana can kill them. This investigation will help the country to make

appropriate policies for malaria treatment and control. For these reasons, we want to collect two teaspoons of blood (3-5 mL x 2) from you who have malaria and isolate the malaria germs from your blood for our investigations. We will also follow up on you on days 1, 2, 3, 7, 14, and 28 to take half teaspoons of blood (1 mL). We are recruiting individuals from the Ewim Urban Health Centre, the Kintampo Municipal Hospital and the Navrongo Municipal Hospital. We decided to choose these places for the study because we know that people who live in these areas, like you, frequently get malaria. Because we are interested in malaria germs, individuals of any group like you can partake in this study. When we take your blood, we will send it to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, and use some special machines to study the malaria germ in your blood and how they respond to the ACTs. Samples would be stored for studying the genetics of the malaria germ deemed necessary under the project. Because some malaria germs are difficult to work with, we would like to keep your sample for up to five years.

Potential Risks and Discomforts

We will collect blood samples from you by venipuncture to study the malaria germs. There may be some common risks, including discomfort, bleeding, or bruising at the spot where the needle enters the body and swelling in the area. Infection and fainting from having blood drawn are usually rare. The site from where the blood will be withdrawn will be adequately clean to prevent any infection. There may be minor pain and discomfort when entering the vein, but the amount of blood that will be taken will not affect your health. A medical doctor will be available to attend in case of any serious injury when taking the blood.

Benefits

Free medical examination and treatment will be made available for you during the study. Individuals diagnosed with malaria will get ACT (either artemether-lumefantrine or artesunate-amodiaquine or dihydroartemisinin-piperaquine) treatment as prescribed by the medical doctor plus appropriate patient care at no cost. There are technicians at the hospital who are assigned to the project, so high quality and rapid diagnostic services will be available to you for early treatment and care.

Cost

Because we will follow up on you to take your blood samples, I would want you to come to the hospital on days 1, 2, 3, 7, 14, and 28. We will re-reimburse you for any financial cost such as transportation due to this study.

Compensation

There will be no compensation for enrolling into the study. However, study participants will be re-reimbursed for any financial cost such as transportation cost due to this study.

Confidentiality

You will not be identified by name in any publication, meeting, abstract, or report derived from the study results or information collected. No information on your genes or heredity will be

attributable to you. Records of patient names and study numbers will be stored in locked and secured computer files and accessible only to key investigators. Individual laboratory results will be made available to clinical personnel involved in the care of patients, the patients themselves, and other caregivers of the patient. All information in paper form will be destroyed after the appropriate holding period.

Funding Information

This project is funded by the University of Edinburg in the United Kingdom and WACCBIP at the University of Ghana.

Voluntary Participation and Right to Leave the Research

Your participation in this study is voluntary, you may withdraw at any time during the study, and access to health care for you will not depend on your participation.

Contacts for Additional Information

1. Dr Yaw Aniweh, WACCBIP, University of Ghana, Legon. Tel: 0207305122
   Email: aniweh@gmail.com
2. Prof Gordon A. Awandare, WACCBIP, University of Ghana, Legon. Tel: 0543717697
   Email: gawandare@ug.edu.gh
3. Dr Lucas Amenga-Etego, Navrongo Health Research Centre, Tel: 0203797997
   Email: lucas.amenga-etego @navrongo-hrc.org

4. Dr Kwaku Poku Asante, Kintampo Health Research Centre, Tel: 0208956598

   Email: kwakupoku.asante@kintampo-hrc.org

5. Felix Ansah, WACCBIP, University of Ghana, Legon. Tel: 0574577600

   Email: fansah004@st.ug.edu.gh

Your rights as a Participant

All the procedures and tests described in this study and any subsequent amendments will be reviewed and approved by an Institutional Review Board (IRB)/Independent Ethics Committee (IEC)/Ethical Review Committee before the commencement of the study.

If you have any questions about your rights as a research participant, you can contact Nana Abena Apatu of Ghana Health Service Ethical Review Committee on Tel: 0503539896 (email: ethics.research.ghsmail.org) between the hours of 9 am - 5 pm from Monday to Friday.

Volunteer Agreement form

The above document describing the benefits, risks and procedures for the research study titled, **the effects of Artemisinin-based combination therapy (ACT) on the dynamics of *Plasmodium falciparum*, *P. malariae* and *P. ovale* infections in Ghana,** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

Name of volunteer: …….....

_____ _____

Date Name and signature or thumbprint of participant

If the participant cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the study volunteer. All questions were answered, and the individuals have agreed to take part in the research.

_____ _____

Date Name and signature or thumbprint of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of person who obtained the consent

ASSENT FORM FOR ADOLESCENTS (12-17 YEARS)

Title: The effects of Artemisinin-based combination therapy (ACT) on the dynamics of *Plasmodium falciparum*, *P. malariae* and *P. ovale* infections in Ghana

Principal Investigator: Prof Gordon A. Awandare

Co-investigators: Felix Ansah (student)

Dr Yaw Aniweh

Dr Emmanuel Amlabu

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Name of volunteer:

……

_____ _____

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If the participant cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the study volunteer. All questions were answered, and the individuals have agreed to take part in the research.

_____ _____

Date Name and signature or thumbprint of witness

I certify that the nature, purpose, potential benefits, and possible risks of participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of person who obtained the consent

PARENTAL CONSENT FORM (1-17 YEARS)

Title: The effects of Artemisinin-based combination therapy (ACT) on the dynamics of *Plasmodium falciparum*, *P. malariae* and *P. ovale* infections in Ghana

Principal Investigator: Prof Gordon A. Awandare

Co-investigators: Felix Ansah (student)

Dr Yaw Aniweh

Dr Emmanuel Amlabu

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P. O. Box LG54, Legon, Accra. Telephone: 0543 717 697. Email: gawandare@ug.edu.gh

General Information about Research

We invite you and your child to participate in a research study that seeks to determine the distribution of malaria germs in Ghana and how these germs respond to the currently used artemisinin-based combination therapies (ACTs) administered in the hospitals. We stopped using some malaria drugs such as chloroquine some years ago because they were no longer killing the malaria germs. Now, ACTs are the best malaria drugs we have. In some parts of the world, it has been reported that the ACTs are also failing to kill some of the malaria germs. Also, there are different malaria germs, and each has a different response to the ACTs. Therefore, it is important to investigate the different malaria germs circulating in Ghana and see if the ACTs given at the hospitals in Ghana can kill them. This investigation will help the country to make appropriate policies for malaria treatment and control. For these reasons, we want to collect two teaspoons of blood (3-5mLx2) from you who have malaria and isolate the

malaria germs from your blood for our investigations. We will also follow up on you on days 1, 2, 3, 7, 14, and 28 to take half teaspoons of blood (1 mL). We are recruiting individuals from the Ewim urban health centre, the Kintampo Municipal Hospital and the Navrongo Municipal Hospital. We decided to choose these places for the study because we know that people who live in these areas, like you, frequently get malaria. Because we are interested in malaria germs, individuals of any group like you can partake in this study. When we take your blood, we will send it to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, and use some special machines to study the malaria germ in your blood and how they respond to the ACTs. Samples would be stored for studying the genetics of the malaria germ deemed necessary under the project. Because some malaria germs are difficult to work with, we would like to keep your sample for up to five years.

Potential Risks and Discomforts

We will collect blood samples from you by venipuncture to study the malaria germs. There may be some common risks, including discomfort, bleeding, or bruising at the spot where the needle enters the body and swelling in the area. Infection and fainting from having blood drawn are usually rare. The site from where the blood will be withdrawn will be adequately clean to prevent any infection. There may be minor pain and discomfort when entering the vein, but the amount of blood that will be taken will not affect your health. A medical doctor will be available to attend in case of any serious injury when taking the blood.

Benefits

Free medical examination and treatment will be made available for you during the study. Individuals diagnosed with malaria will get ACT (either artemether-lumefantrine or artesunate-

amodiaquine or dihydroartemisinin-piperaquine) treatment as prescribed by the medical doctor plus appropriate patient care at no cost. There are technicians at the hospital who are assigned to the project, so high quality and rapid diagnostic services will be available to you for early treatment and care.

Cost

Because we will follow up on you to take your blood samples, I would want you to come to the hospital on days 1, 2, 3, 7, 14, and 28. We will re-reimburse you for any financial cost such as transportation due to this study.

Compensation

There will be no compensation for enrolling into the study. However, study participants will be re-reimbursed for any financial cost such as transportation cost due to this study.

Confidentiality

You and your child will not be identified by name in any publication, meeting, abstract, or report derived from the study results or information collected. No information on your genes or heredity will be attributable to you. Records of patient names and study numbers will be stored in a locked file and secured computer files, and accessible only to key investigators. Individual laboratory results will be made available to clinical personnel involved in the care of patients, the patients themselves, and other caregivers of the patient. All information in paper form will be destroyed after the appropriate holding period.

Funding Information

This project is funded by the University of Edinburg in the United Kingdom and WACCBIP at the University of Ghana.

Voluntary Participation and Right to Leave the Research

Your participation in this study is voluntary, you may withdraw at any time during the study, and access to health care for you will not depend on your participation.

Contacts for Additional Information

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2. Prof Gordon A. Awandare, WACCBIP, University of Ghana, Legon. Tel: 0543717697
   Email: gawandare@ug.edu.gh
3. Dr Lucas Amenga-Etego, Navrongo Health Research Centre, Tel: 0203797997
   Email: lucas.amenga-etego @navrongo-hrc.org
4. Dr Kwaku Poku Asante, Kintampo Health Research Centre, Tel: 0208956598
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5. Felix Ansah, WACCBIP, University of Ghana, Legon. Tel: 0574577600
   Email: fansah004@st.ug.edu.gh

Your rights as a Participant

All the procedures and tests described in this study and any subsequent amendments will be reviewed and approved by an Institutional Review Board (IRB)/Independent Ethics Committee (IEC)/Ethical Review Committee before the commencement of the study.

If you have any questions about your rights as a research participant, you can contact Nana Abena Apatu of Ghana Health Service Ethical Review Committee on Tel: 0503539896 (email: ethics.research.ghsmail.org) between the hours of 9 am - 5 pm from Monday to Friday.

Volunteer Agreement form

The above document describing the benefits, risks and procedures for the research study titled, **the effects of Artemisinin-based combination therapy (ACT) on the dynamics of *Plasmodium falciparum*, *P. malariae* and *P. ovale* infections in Ghana,** has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to allow my child/ward to participate as a volunteer.

Name of child: …….....

_____ _____

Date Name and signature or thumbprint of parent or guardian

If parent/guardian/participant cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the parent/guardian of the child. All questions were answered, and the individual or parent/guardian has agreed to allow his/her child to take part in the research.

_____ _____

Date Name and signature or thumbprint of witness

I certify that the nature, purpose, potential benefits, and possible risks of participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of person who obtained the consent

ENROLLMENT FORM FOR ADULTS (18 YEARS AND ABOVE)

DATE: ____/_____/_____ **Study ID_____**

(Day / month / year)

1. Personal Details

1.1 Name of Participant:

……

1.2 Study ID. No: ……

1.3 Date of Birth: ……/……../……

(Day / month / year)

1.4 Age (yrs) ……

1.5 Was weighing card or birth certificate available? Yes=1 No=2

1.6 Sex: Male=1 Female=2

1.7 Informed consent obtained: Yes= 1 No=2

1.8 Community of residence……

1.9 Duration of residence in the community ……

2. History of illness

2.1 When was the last time the participant was ill? *Please indicate*……

2.2 What was the suspected illness? .....

2.3 Was the participant taken to health centre/hospital? Yes=1 No=2

2.4 Has the participant taken any anti-malarial drug in the last two weeks? Yes=1 No=2

2.5 Did the participant take any traditional or herbal medication? Yes=1 No= 2

2.6 Indicate which of the following the participant felt or showed? *Please indicate Yes or No*

2.6.1 Fever Yes=1No=2

2.6.2 Headache Yes=1 No=2

2.6.3 Nausea/Vomiting Yes=1 No=2

2.6.4 Chills/Rigor Yes=1 No=2

2.6.5 Joint pains Yes=1 No=2

2.6.6 Diarrhoea Yes=1 No=2

2.6.7 Convulsions Yes=1 No=2

2.6.8 Jaundice/Deep yellow eyes Yes=1 No=2

symptoms……

2.7 How many times in a year has the participant been diagnosed with malaria (approx.):

2.8 Body temperature (axillary) at visit: ……^{o}C

2.9 Weight in **Kg**……

2.10 Height in **cm**……

2.11 a) Sickle cell trait status known? Yes=1 No=2

b) If known, what is the genotype? Normal (AA)=1 Carrier (AS)=2 Sickling (SS)=3 Others=4

2.12 Does the participant regularly sleep under a bed net? Yes=1 No=2

3. Socio-Economic Background

3.1 Educational background:

None=0 Primary=1 Middle=2 JSS=3 Vocation=4 Secondary=5 Tertiary=6

3.2 Occupation: Housewife=1 Farmer=2 Teacher=3 Trader=4 Other = 5

3.3 How many individuals do you have in your household? *Please indicate*: 1, 2, 3, 4, 5, ≥ 6

3.4 Indicate which of the following you own

3.4.1 Cement block house Yes=1 No=2

3.4.2 Thatched house Yes=1 No=2

3.4.3 Car(s) Yes=1 No=2

3.4.4 TV set Yes=1 No=2

3.4.5 Radio set Yes=1 No=2

3.4.6 Farm Yes=1 No=2

3.4.7 Fridge Yes=1 No=2

4.1 Name of interviewer: ……..

Signature/code: ……..

ENROLLMENT FORM (1-17 YEARS)

DATE: ____/_____/_____ **Study ID_____**

(Day / month / year)

1.0 Personal Details

1.1 Name of Participant: ……

1.2 Father's name: ……

1.3 Mother's name: ……

1.4 Study ID. No: ……

1.5 Date of Birth: ……/……../……

(Day / month / year)

1.6 Age (yrs) ……

1.7 Was weighing card or birth certificate available? Yes=1 No=2

1.8 Sex: Male=1 Female=2

1.9 Informed consent obtained: Yes= 1 No=2

1.10 Informed consent given by: Parent=1 Guardian=2 Participant=3

1.11 Community of residence ……

1.12 Duration of residence in the community ……

2.0 History of illness

2.1 When was the last time the participant was ill? *Please indicate*……

2.2 What was the suspected illness? .....

2.3 Was the participant taken to health centre/hospital? Yes=1 No=2

2.4 Has the participant taken any anti-malarial drug in the last two weeks? Yes=1 No=2

2.5 Did the participant take any traditional or herbal medication? Yes=1 No= 2

2.6 Indicate which of the following the participant felt or showed? *Please indicate Yes or No*

2.6.1 Fever	Yes=1 No=2
2.6.2 Headache	Yes=1 No=2
2.6.3 Nausea/Vomiting	Yes=1 No=2
2.6.4 Chills/Rigor	Yes=1 No=2
2.6.5 Joint pains	Yes=1 No=2
2.6.6 Diarrhoea	Yes=1 No=2
2.6.7 Convulsions	Yes=1 No=2
2.6.8 Jaundice/Deep yellow eyes	Yes=1 No=2

2.6.9 Please specify any other symptoms……

2.7 How many times in a year has the participant been diagnosed with malaria (approx.)....……

2.8 Body temperature (axillary) at visit: ……^{o}C

2.9 Weight in **Kg**…….

2.10 Height in **cm**……

2.11 a) Sickle cell trait status known? Yes=1 No=2

b) If known, what is the genotype? Normal (AA)=1 Carrier (AS)=2 Sickling (SS)=3 Others=4

2.12 Does the participant regularly sleep under a bed net? Yes=1 No=2

3.0 Socio-Economic Background

3.1 Mother's educational background:

None=0 Primary=1 Middle=2 JSS=3 Vocation=4 Secondary=5 Tertiary=6

3.2 Mother's occupation: Housewife=1 Farmer=2 Teacher=3 Trader=4 Other = 5

Other please specify……

3.3 Father's educational background:

None=0 Primary=1 Middle=2 JSS=3 Vocation=4 Secondary=5 Tertiary=6

3.4 Father's occupation: Farmer=1 Teacher=2 Trader=3 Other = 4

Other please specify……

3.5 How many children do you have in your household? *Please indicate*: 1 2 3 4 5 ≥ 6

3.6 How many siblings does the child have? *Please indicate*: 1 2 3 4 5 ≥ 6

3.7 Indicate which of the following owned by either mother or father or guardian

3.7.1 Cement block house Yes=1 No=2

3.7.2 Thatched house Yes=1 No=2

3.7.3 Car(s) Yes=1 No=2

3.7.4 TV set Yes=1 No=2

3.7.5 Radio set Yes=1 No=2

3.7.6 Farm Yes=1 No=2

3.7.7 Fridge Yes=1 No=2

4.1 Name of interviewer: ……..

Signature/code: ……..

ENROLLMENT LAB FORM

Study ID. …….

Date: ……/……/…….

(Day / month / year)

1.0 Venous blood sample taken? Yes=1 No=2 ☐

1.2 Filter paper sample obtained? Yes=1 No=2 ☐

1.3 Thick and thin blood smears made? Yes=1 No=2 ☐

1.4 RDT test performed? Yes=1 No=2 ☐

1.5 Hb measured? Yes=1 No=2 ☐

If yes, please indicate value ……g/dl

1.6 Sickle cell status ……..

LABORATORY RESULTS

Blood film code……

Comments: ……..

= no parasites, 1 = *P. falciparum*, 2 = other, 3 = mixed

Parasite Count/200WBC……

WBC count……

Parasite Density……..… /µl blood

RDT test- Positive ☐ Negative

Clinical Diagnosis:

= unconfirmed malaria, 2 = confirmed malaria, 3 = asymptomatic parasitaemia,

9 = other, 0 = no disease). ☐

Obvious clinical evidence of bacterial infection (1= Yes, 2= No) ☐

Diagnosed by . :……

Signature/code: …… Date: ……/……/……

(Day / month / year)